Vol. XXV. *September, 1913.* *No. 4*

BIOLOGICAL BULLETIN

A STUDY OF THE EFFECTS OF INJURY UPON THE FERTILIZING POWER OF SPERM.

NEIL S. DUNGAY.

CONTENTS.

Page.

I. Introduction 213
II. Materials and general methods 214
A. Occurrence 214
B. Collecting 214
C. Removal of germ cells 215
D. Culture methods 215
E. Controls 216
F. Treatment of sperm cells 216
G. Cytological methods 217
III. Outline of development of *Nereis* eggs 218
IV. Experiments 220
A. Upon *Nereis* 220
1. Heat 220
2. Delay 227
3. Fresh water 229
4. Alcohol 230
5. Sodium hydroxide 231
6. Hydrochloric acid 232
7. Cold 233
B. Upon *Arbacia* 233
V. Cytological study 236
A. Normal 236
B. Experimental 237
VI. Discussion 244
A. The production of defectives 244
B. Fertilization 252
VII. Summary 254

I. INTRODUCTION.

This paper deals with the results obtained by fertilizing normal eggs with spermatozoa injured by various methods. The results have a bearing upon some of the problems of fertilization.

They also throw light upon the theories dealing with the production of defectives by the action of injurious agencies upon the germ cells previous to the time of fertilization.

The experimental work was done at the Marine Biological Laboratory at Woods Hole, Mass., during the summers of 1911 and 1912. The results given are based upon observations made upon more than 600 cultures of living *Nereis* eggs and 200 cultures of *Arbacia* eggs. Samples from the various cultures were preserved for later cytological study, the results of which are also reported in this paper.

II. Materials and General Methods.

A. Occurrence.

In the evening from the time of darkening until about ten o'clock, sexually mature males and females of *Nereis limbata* swim freely at the surface of the Eel Pond at Woods Hole. Although they are most easily collected during the two weeks following the full moon, it is possible to find a few nearly every evening of the lunar month. The breeding habits have recently been described (Lillie and Just, '13) in a very interesting paper which gives in considerable detail the methods of catching and keeping the worms.

B. Collecting.

For my experiments the animals were caught in nets by lantern light, placed in separate finger bowls of sea water, and taken to the laboratory buildings at about 9:30 P.M. The worms were then washed in running sea water and the finger bowls were cleansed and refilled, covered with glass plates and placed in running water to keep cool. Early the next morning the accumulations of mucus were removed and the water was again changed. When carefully cared for in this manner, very few of the worms shed their germ cells during the night. The males practically never shed their sperm cells under these conditions. During one month in which a record was kept, over 90 per cent. of the females captured retained their eggs. By this method I have sometimes kept the females without shedding for as long as 60 hours, though, for experimental purposes, the eggs

which were retained so long were not used. The experimental work was usually started as early in the morning as possible so as to use the material in a fresh condition. A few experiments were carried out shortly after removing the animals from the Eel Pond. Since no better results were secured by working at night, the material was usually kept until morning when conditions for work were better.

C. Removal of Germ Cells.

The abundant eggs and sperm were removed by snipping the sides of the body wall at intervals, with scissors. If no water is used the sperms push out as a white mass nearly free from liquid and the eggs may be secured as a thick greenish mass. As a rule, however, the operation was conducted under water. In all cases the usual precautions were taken to avoid the introduction of stray sperm cells. The worms were thoroughly washed in a jet of salt water and, in the earlier experiments, in boiled salt water as well, a precaution which I afterwards found to be unnecessary since the sea water from the laboratory supply was repeatedly tested and found to contain no active *Nereis* sperm. The bodies of the animals used were quickly removed from the dishes of sperm and eggs so as to avoid, as far as possible, any contamination from body fluids, and every precaution was taken to avoid abnormalities due to outside influences such as strong sunlight, bacterial action, mechanical agitation, etc.

D. Culture Methods.

An effort was made to use a minimal quantity of sperm for the process of insemination, in order to avoid, as far as possible, errors due to polyspermy. In the case of the injured sperm, a large quantity was often necessary to secure a small percentage of fertilized eggs. Usually a small number of eggs was inseminated first in a small quantity of water to which a liberal supply of water was soon added. In this manner I was able to secure a sufficient number of fertilized eggs to make preservation possible. Up to the time when the larvæ begin to swim, the water was changed several times. After this period it is very difficult to renew the water without losing many of the larvæ. An effort

was made to keep from crowding the developing eggs since this seems to be a source of abnormality. Any error which might creep in here was checked by the controls since they always contained at least as many eggs as were kept in the experimental cultures.

E. Controls.

Along with each experiment two or more control cultures were kept. One control consisted of a dish of sea water containing a part of the eggs which were not inseminated. The other contained about the same number of eggs as was kept in each of the experimental dishes, inseminated with injured sperm; these were as closely followed during the period of observation as were the experimental cultures. In all experiments in which the sperm cells were subjected to the action of reagents it is impossible to avoid carrying over a trace of the reagent used, to the dishes of eggs. Control experiments were made in such cases by adding to the eggs at the time of insemination, as much of the reagent as was introduced into the experimental cultures. In nearly all cases the eggs and sperm used in the controls came from the same animals which furnished the materials for the experiments.

F. Treatment of Sperm Cells.

Attempts to injure the sperm cells as much as possible without preventing their attachment to the eggs proved unsuccessful in many cases. Either the sperm were killed or they were not sufficiently affected to give striking results. With improvement of the technique consistent results were secured, although these results indicated a great variation in the intensity of the injuries to the sperm cells, thus rendering it impossible to repeat an experiment with a complete duplication of results.

The sperm cells were injured in various manners. Inasmuch as some methods gave promise of easier and more striking results or seemed less open to criticism, but few experiments were made along other lines in order to give more time to follow out the more desirable methods. Injury was attempted by soaking the sperm cells in distilled water or in diluted sea water; by treating them with weak alcohol; by exposing them to the action of dilute acids or alkalis; by freezing; by heating; and

by keeping them so long that they were nearly dead. Since it proved to be so difficult to injure the sperm without destroying their motility it seemed to be desirable to try the effects of the X-rays or of the radium emanations; but as the proper appliances were not procurable at the time, I was unable to carry out this part of the work. An attempt was made to get results by using sunlight but the sperm cells gather together in such a way that they protect each other. No attempt was made to carry this method far although it might prove useful with proper technique.

G. Cytological Methods.

For later cytological study of the early stages, a large number of samples were taken from the experimental cultures and fixed in Meves's modification of Flemming's fluid, made as follows: 3.5 c.c. osmic acid, 2 per cent.; 15 c.c. chromic acid, 0.5 per cent.; 3 drops glacial acetic acid. The eggs were exposed to the action of this solution for about 40 minutes. Iron hæmatoxylin was used as a stain. The larval stages were preserved either in Gilson's mercuro-nitrate fixing fluid or in Bouin's mixture of formalin and picric acid.

The fact should be mentioned at this point that it was found to be impossible for one person to make as careful observations and notes upon the living material, when preserving a series for later study, as were made when doing nothing else except following the developing eggs under the microscope. As a consequence the preserved samples are of two kinds, viz.: (1) a long close series with as much recorded data as the time allowed, and (2) irregular preservations made during extended periods of observation. Usually in getting a long series fixed it was necessary, especially in the earlier stages, to run at least two series at one time, since it is impossible to tell with certainty just how much the sperm cells used for inseminating have been injured. Again it should be noted that in the preservation of a long series, those samples first fixed often contain a greater proportion of the more normal eggs than those preserved later. This is due to the fact that the most normal sperm cells seem to initiate development earlier than do the others. Since the eggs which have formed jelly are more readily picked up by the pipette,

they will tend to be taken from the cultures in greater proportions in the earlier part of the preserved series. An effort was made to overcome this by using several parallel cultures in different dishes but without entire success since the number of eggs from a single female is limited and since it does not seem wise to use eggs from two or more females, in the same experiment.

III. Outline of Development of Nereis Eggs.

Although the maturation, fertilization, and development of the egg of *Nereis* have been described in more or less detail by others (*e. g.*, Wilson, '92, Lillie, F. R., '11, and '12) it seems desirable to review a few of the principal points at this time. The eggs, which vary considerably in size, are about 100 microns in diameter. Their irregular shape at the time of laying is soon lost and they take the form of a sphere slightly compressed in a polar direction. They are of a greenish color and moderately transparent. A polar view, which is the one usually obtained, shows the large germinal vesicle in the center of the egg and an imperfect double belt of large oil drops, numbering approximately from ten to twenty. Just beneath the vitelline membrane is a radially striated cortical layer which breaks down immediately following insemination, throwing off a thick envelope of jelly and leaving a conspicuous perivitelline space. The jelly is readily demonstrated by the use of India ink ground up in sea water, although it is nearly invisible in pure sea water. Since the unfertilized eggs normally lie in contact at the bottom of a dish and, upon insemination, become separated by the jelly, it is easy for the naked eye to determine whether or not a large part of the eggs have started to develop. A microscope, however, is necessary to determine the condition of any individual egg. As a general rule unfertilized eggs do not form jelly upon standing undisturbed, at least not for several hours. Yet close examination usually reveals a mere trace of eggs which have formed this secretion. Inasmuch as strong mechanical agitation will induce jelly formation this is perhaps due to violent contact with the scissors upon opening the female, or possibly there may be a few eggs which are extraordinarily sensitive to stimulation. The latter view is rendered probable by the fact that the pro-

portion of eggs with jelly varies considerably among different lots, so much so that an occasional batch of eggs is found in which the majority form jelly. This jelly formation is probably not due to accidental insemination since fertilization cones are not seen and cleavage does not result. Yet to guard against possible misinterpretation an unfertilized control was kept for each experiment and examined along with the experimental materials.

The active spermatozoön may be seen to attach itself to the egg. It immediately becomes quiescent and remains motionless until it is drawn into the egg. The outflow of jelly, which continues for some fifteen minutes after insemination, now sweeps away all superfluous sperm cells, which have in the meantime lost their power of motion. Just beneath the attached sperm cell an attraction cone is pushed up by the egg cytoplasm until it unites with the membrane. It then gradually disappears, carrying with it the membrane so as to form a depression in which the sperm is now concealed. This all happens during the first 25 minutes after insemination. In the meantime the germinal vesicle has disappeared and the egg has assumed an irregular shape. About 45 minutes after insemination the egg resumes its spherical form and soon the sperm, after again coming prominently into view for a short time, is drawn into the egg. Shortly before the close of the first hour succeeding insemination the first polar body appears and is followed in a few minutes by the second. The polar bodies are easily seen at this time against the now yolk-free region of the pole. In a profile view they are very conspicuous. In the course of the second hour the first two planes of cleavage appear. The first plane, as has been stated by Just ('12), passes through the point of entrance of the sperm, dividing the egg unequally. The development proceeds rapidly from this point. Movement is often seen in twelve hours and fine trochophores are formed by the end of the first day. In the second day the animal begins to elongate and the larval setæ grow out to a relatively great length. Naturally the time element, as given above, varies greatly with the temperature.

IV. Experiments.

The sexual products of both *Nereis* and *Arbacia* were utilized for experimental purposes. However at the time when I could use *Arbacia*, the control cultures did not run as well as might be desired and so *Nereis* material was used for the greater part of the work.

A. Upon Nereis.

In the earlier experiments upon *Nereis* I subjected the sperm cells for a certain time to the action of graded strengths of various reagents. Later I found that the most satisfactory procedure is to keep the sperm cells, after removal from the body of the male, until they are nearly dead or to keep them for a few minutes at a temperature of about 44° C. The latter method seems to be less open to criticism since no substances of a possibly toxic action are transferred to the egg cultures by the process of insemination.

All of the methods of experimentation which were used give essentially similar results, indicating that the action upon the sperm cells is probably not specific in any case. It seems rather that there is produced a general decrease in vitality which may manifest itself by a retarded development, by a high mortality or by the production of forms which are more or less abnormal in structure or behavior.

1. *Heat.*—Perhaps the most uniform results in the whole series of experiments which I have carried out were obtained through the exposure of the sperm cells to a certain degree of heat. This was accomplished by mixing the sperm cells with sea water, which was then placed in the bottom of a test tube, care being taken to prevent any of the liquid from touching the walls of the upper part of the tube. A thermometer was then introduced into the bottom of the test tube and was used to stir the liquid and to take the temperature readings every minute. The test tube was nearly immersed in a large beaker of warm water. The temperature was maintained in the bath thus formed, by means of an alcohol lamp or by adding to the bath warmer water from another container. In this manner it is possible to keep the temperature under reasonable control.

Particular care was taken to insure the perfect cleanliness of all pipettes. None of the liquid of the test tube was allowed to splash upon the sides of the tube so as to be above the level of the water in the bath. A large number of dishes of eggs were made ready and one or more of these were inseminated every minute until either all the sperms had been used up or the dishes of eggs had all been inseminated. In this way there were usually obtained one or more dishes of eggs which were inseminated at the critical time which occurred from 7 to 20 minutes after the sperm cells were placed in the warm bath. The variation in time is probably due to fluctuations in the temperature of the bath, small though they may be. This is borne out by some experiments in which slightly higher temperatures were used and in which the critical time came much earlier, so early in fact that it was difficult to catch the sperm cells at just the proper stage for use.

Since it proved to be very difficult to determine in advance which culture would be the best for study, the preservation of a long series from the best cultures is largely a matter of chance. But by preserving a large number of series for cytological examination, a few have been obtained which are satisfactory for study. The others, although they show essentially the same features, contain so many eggs which develop normally that the study of the affected eggs is very time consuming.

In all, 23 experiments, involving several hundreds of cultures, were made by this method. Since the earlier work was carried out in a different manner, which gave me records of the course of development of the experimental cultures but no continuous series of preserved samples, the primary purpose of this set of experiments was to obtain material for cytological work. Accordingly the records of these experiments are not as full as might be desired in the case of any one experiment.

To illustrate the method of procedure the record of experiment 65 of July 4, 1912 is given. The eggs were removed from the female at 1:39 P.M. Sperm cells removed from male at 1:40 P.M. Sperm placed in bath at 1:47 P.M. Temperature of bath at end of successive minutes (read from centigrade thermometer in test tube) 43°; 44°; 43.4°; 43°; 44°; 44°; 43.8°; 43.3°;

43°; 43.7°; 43°; 43.7°; 43°; no later records. Samples of eggs from one female, numbered 65.1, 65.2, 65.3, 65.4, and 65.5, were inseminated with heated sperm at the end of 11, 12, 13, 15, and 16 minutes respectively. In the first four about 95 per cent. of the eggs formed jelly immediately. Sample 65.5 gave about 80 per cent. with jelly. All of the above were discontinued. 65.6 was inseminated at 2:04 P.M. with sperm which had been heated for 17 minutes. Jelly formation began at once but took place in a gradual manner, some eggs failing to give off jelly until the end of half an hour. Eventually about 60 per cent. formed jelly. Of the eggs with jelly 50 per cent. failed to segment, 15 per cent died before gastrulation, 25 per cent. formed trochophore larvæ later than did the controls, and 10 per cent. behaved in a seemingly normal fashion. Discontinued before the formation of the larval setæ because all had been preserved. Control of infertile eggs showed only a trace of jelly formation after 18 hours. The inseminated control developed normally. A series of preservations from the experimental culture 65.6 was made at 10 minute intervals, beginning at 2:24 P.M.

Experiment 63, July 3, 1912, was conducted in a similar manner and furnishes a somewhat fuller record of the later stages. 63.5 was inseminated at 10:36 with sperm which had been kept in a warm bath (about 44° C.) for 14 minutes. Over 70 per cent. formed jelly. First cleavage was observed at 12:15 P.M., 7 minutes later than in the case of the control series. At 1:25 P.M. the control culture was nearly two cleavages ahead of the experimental culture. About 5 per cent. of the eggs in the experimental culture failed to segment after forming jelly. At 8:45 A.M. on the following day the experimental culture was very much less active than the control. About 5 per cent. had died in cleavage stages. 49 hours after insemination the control animals were in excellent condition and were sending out the second set of larval setæ. The experimental culture showed clearly that the injury to the sperm cells may cause abnormalities in those eggs which seem to behave normally in the earlier stages. 50 per cent. of the developing eggs had not gone beyond a trochophore stage. Most of these died soon, though a few continued to live for at least another day without any change except an

increase of size. This growth is probably due to an increase in water content and gives the animals a dropsical appearance. 25 per cent. were as well developed as the control animals. The remaining 25 per cent. were in various stages from the trochophore to the formation of the second set of larval setæ. Some of them seemed to be normal in every way except that their development was retarded. Others were less active. A small number, perhaps 5 per cent., seemed to be as far along as the controls but had no setæ or were deficient in some other way. Most of these died within a few hours. The controls developed in a normal manner. The uninseminated controls showed no development beyond the formation of jelly in about 0.3 per cent. In all the cultures of the above experiment the conditions were made as good as possible by changing the water, removing dead eggs, etc.

Without attempting to give other definite records, I may summarize briefly the different types of effect observed in the series of experiments using heat as a means of altering the sperm cells.

(*a*) In some cases all of the jelly did not form and the peripheral alveoli of the egg were not entirely emptied. This occurred in from a trace to nearly 10 per cent. of the eggs in the experimental cultures. Some eggs formed a mere trace of jelly, some formed about half of the normal quantity, and some had nearly as much as normal. The different cases observed gave the impression that a larger stimulus caused a greater jelly formation. It seemed as though a sperm might in some way give only enough impulse to cause a part of the alveoli to be emptied, possibly due to a brief attachment to the egg. However no such egg was ever followed long enough to determine the cause of this partial jelly formation.

(*b*) After the jelly is completely extruded and the polar bodies have been formed, cells are commonly found in which no further development takes place. A few eggs do this in many uninseminated controls but there is no comparison in the frequency with which they may be found. In some experiments special care was taken to avoid mechanical agitation since violent mechanical stimuli may produce this effect. Yet the same

results were found, indicating that maturation and jelly formation had been initiated by the sperm in some way, although later development failed to take place. In some experiments this has been seen in more than half of the eggs which formed jelly. All experiments which gave any indication of abnormality contained at least a few of this type of eggs. In those cultures in which these eggs were present in considerable numbers I failed to see as many fertilization cones as usual. This suggests either that the eggs were infertile, due to the failure of the sperm to enter, or that the development was carried this far in response to some unknown stimulus given at the time of insemination. Cytological examination is necessary to determine this point.

(*c*) Cytological examination of stained sections showed cases in which polar bodies appeared without jelly formation. No indication of this was seen in the living material as it was not supposed that it was possible. It would, of course, be difficult to find, even if such cases were known to occur.

(*d*) Development sometimes stopped after the first cleavage or more rarely after the second. This occurred in less than 2 per cent. usually. It was usually associated with

(*e*) an unsuccessful attempt to form a first cleavage plane. The behavior of such cases will be described under another heading in more detail.

(*f*) In some cases, as many as 0.5 per cent. in one experiment, the first cleavage plane divided the cell into two equal parts. Such cases did not usually divide again.

(*g*) In a very few egg cells the cleavage was uneven, forming 3, 5, or 7 cell stages which usually died without developing far. It is well known that uninseminated eggs may undergo a kind of pseudo-cleavage if allowed to stand for some hours but the cases mentioned here are not to be confused with this for several reasons. Their appearance is different. These forms appear much earlier than they ever do in the unfertile eggs. The control of unfertilized eggs never showed such pictures until much later and then they were of a different appearance.

(*h*) Other irregular forms of cleavage were seen, but very rarely. Since the later cleavage stages are more complex in appearance it is more difficult to find irregularities and they

may be of more frequent appearance than my notes indicate. Again it may be possible that such cases are sometimes present in eggs fertilized with normal sperm. I have no reason to believe that my control cultures contained such cases.

(*i*) The rate of development in the experimental cultures often proved to be very irregular and was usually slower than that of the controls. For example, when the controls first have definite setæ the experimental cultures have none. Segments are marked out earlier in the controls. Early cleavage planes are often delayed, but this may be due in some part to the failure of the weakened sperm to reach the egg as soon as it should. But it seems certain that, even allowing for this fact, the early stages are somewhat retarded in development.

(*j*) In practically every experiment the controls lived longer than the experimental cultures and the death rate in them was not so much as one tenth as large. Although an effort was made to keep the controls under exactly the same conditions as the others there were usually fewer larvae in the experimental dishes (presumably a more favorable condition). Old eggs and organic materials were removed so as to eliminate any possible toxic action of products of decay. In some cases the water was changed from day to day but with little effect. It seems that the normal animals in the controls are able to withstand the artificial conditions incident to life in the laboratory for a much longer time than those in the experimental cultures, which are so weakened by the injury to the part which comes from the male parent, that they cannot exist under such conditions as are provided.

(*k*) The trochophore stage seems to be a difficult one to pass. It frequently happens that this stage is permanent. In some cases at about the time when segments should be marked out and setæ should appear, the animal becomes irregular in form or swells up and becomes transparent, due probably to the absorption of water. In some cases the trochophore remains in its original condition without change. In most cases death takes place soon after elongation should take place but I have often kept the permanent trochophores for a day or more after they should have elongated.

(*l*) When the larval setæ should form, it often happens that none or only a few of them appear. In some cases this was true of more than half of the larvæ. Very few experimental cultures showed less than 5 per cent. of such forms. There may be all grades of conditions from no setæ, through a few scattering ones, weak ones, and abnormally directed ones, to nearly perfect forms. In cultures inseminated with normal sperm, very adverse external conditions may produce this effect or something, in a small number of the larvæ, very much like it. But under the very best conditions that could be maintained this was always found in the experimental material, though never in the controls. Associated with this condition was the partial or complete lack of sensory appendages.

(*m*) The body form is sometimes altered. It may be permanently bent toward one side by the asymmetrical development of a single segment. Often the body is covered with small wart like projections, giving it a peculiar roughened appearance.

(*n*) The experimental cultures nearly always show a lack of vitality, as is evidenced by a lessened activity. The normal cultures are very active after reaching the motile stages. The others, at least in part, are usually more or less quiescent. The phototropic responses are much less uniform in the experimental cultures and do not take place so quickly.

(*o*) In addition to the above list of abnormalities there is always present, unless the unfertile eggs are removed from the cultures, a number of other types which are confusing at first. As it is not always possible to remove every unfertilized egg during the first few hours, one is likely to find a variety of things which are easily misinterpreted unless they are very carefully studied. Unless such things have crept into my counts through an occasional oversight no consideration will be given them.

Nearly all of the above list of abnormalities were present in most of the experimental cultures if they were not discontinued too early. The controls rarely produced any of them. The proportions of the different types naturally differed widely in the various experiments. If the sperm cells were injured to the limit, or nearly so, the abnormalities were largely confined to the early stages and death occurred before the later ones could ap-

pear, though a few individuals nearly always succeeded in reaching the later stages. If the sperm cells were not injured so much, the earlier stages were comparatively free from abnormalities and those of the later stages appeared, chiefly the permanent trochophores, the malformed bodies and the lack of setæ.

In these experiments, as in those to be described later, the number of eggs fertilized is inversely proportional to the strength and duration of action of the injurious agent, provided the proportions of eggs and sperm are constant. The degree of abnormality and the per cent. of abnormal forms is directly proportional to the strength and duration of action of the agent used. These statements, of course, are not to be taken as more than an approximation. No effort has been made to reduce the results to the form of an exact law. However the results of the series are consistent and conform nicely to the above generalizations.

2. *Delay.*—Striking results were obtained by keeping the sperm cells for some time before they were used for insemination. Naturally the extreme time which may elapse before the sperm cells become incapable of influencing the eggs varies greatly with the temperature, with bacterial conditions, and, I suspect, with the general vitality of the cells. The complexity of the conditions makes it extremely difficult to completely duplicate the results of any particular experiment. Oftentimes it happens that the results in experimental cultures are nearly normal and again development may not appear at all. Yet the results all seem to be consistent, the differences being in degree only. As in the set of experiments described under the head of heat, the abnormal types are undoubtedly due to certain degrees of injury to the sperm cells. The possibility of the results being due to toxic substances produced by bacterial action or sperm metabolism during the period of delay, and introduced into the egg cultures at the time of insemination, seems to be rather remote. In order to minimize any such effect, the water was changed on the egg cultures several times shortly after insemination.

One of the several extreme cases of effect upon early development is found in experiment 34 of July 15, 1911. The uninseminated control remained unchanged. The fertilized control was inseminated at 2:11 P.M. At 3:15 P.M. the first cleavage was

practically completed and many eggs were dividing the second time. At 3:25 the majority of the eggs were in a 4 cell stage. At 9:00 A.M. of the following day all were normal trochophores. The second day brought forth setæ and succeeding days showed only a normal development. 34.1 consisted of fresh eggs and a large quantity of sperm which had been kept in a corked vial for 21 hours at room temperature. The sperm and eggs were mixed at 1:35 P.M. Less than 5 per cent. of the eggs formed jelly. At 2:59 P.M. there were many attempts to form a two cell stage. It will be noticed that this is over 20 minutes longer after insemination than the time at which the controls were nearly through with the first cleavage. Very few normal 2 cell stages resulted. Usually the constriction appeared in the proper location but a few cases were observed in which the attempted plane of cleavage would have divided the cell into equal parts, had it been completed. In the great majority of cases the constriction lasted for a few minutes and then began slowly to disappear, the cell becoming irregular in outline. For example one cell was observed to be in the early process of first cleavage at 3:18 P.M. Slight progress was made for a minute and then no change took place until 3:23 P.M. when the furrow began to disappear. At 3:37 P.M. the furrow was invisible and there was no trace of the attempted cleavage. Irregularities in cleavage were also seen. At 3:55 P.M. a 5-cell stage was noted. A 4-cell stage with two very large cells diametrically opposite to each other was observed at the same time. Another distinct 5-cell stage was seen. Another 4-cell stage, as seen from a polar view, had cells of equal size, the polar bodies appearing to be normal. At 3:40 P.M. and following, another case of attempted cleavage, similar to the one described above, was seen. At 3:44 P.M. a 3-cell stage was found. Many one cell stages which had formed jelly and thrown off polar bodies were now evident. It was not determined whether many of these had previously attempted to divide or not. Probably most of them had not. At 3:45 P.M. a 7-cell stage was seen. At 9:00 A.M. of the following day most of the eggs with jelly were still in a one cell stage or in irregular early cleavage. Much of this irregular early cleavage is probably not a true cleavage but a

budding. About 4 per cent. of the eggs were in a 2-cell stage of apparently normal form. A few trochophores, not at all active, were revolving feebly with a lateral displacement of perhaps twice their own diameter. They were abnormal in shape and appearance and though no cilia could be seen they were certainly present since the animals were motile. The whole culture died without developing farther. Other experiments gave similar results or produced fewer abnormalities in early development and lived to produce malformed larvæ.

Since on one day sperm which had been kept for 30 hours would cause most of the eggs in a culture to develop and upon the following day sperm only half as old might have no effect it seemed to be desirable to produce more uniform conditions for keeping the sperm. Accordingly the sperm cells were placed in an ordinary refrigerator in which the comparatively low temperature did not fluctuate so much as did that of the outer air. The best results were secured by using the sperm on the third day. By testing out the contents of a vial at intervals during the day it is possible to determine the proper time for starting an experiment. About 40 cultures obtained in this way have been under observation and have given reasonably uniform results. All of the types of abnormal development described above under the head of heat were repeatedly found in this set of experiments. The results given by this set of experiments are very convincing to one who was able to follow the material closely. It is not known definitely whether the injury to the sperm was produced by toxic bacterial products, by the accumulation of metabolic products, or by the gradual weakening of the cell by its own metabolism. Whatever the cause, I have no doubt that the effects produced were brought about by insemination with weakened sperm cells.

3. *Fresh Water.*—Three experiments were made in which the sperm cells were placed in dilute sea water or in distilled water for short periods of time. Sperm from mixtures containing more than 20 per cent. of sea water produced normal development. The results produced by lower percentages of sea water and by distilled water were variable, some normal larvæ appearing in cultures in which most of the eggs failed to segment. Permanent

one and two cell stages were secured in large numbers. Attempts at first cleavage were also seen. Although so few experiments were made by this method that we do not get all the types of abnormality which were produced by the heat and delay, we have a clear indication that more work and better technique would produce very similar results. The work was dropped in order to take up more promising methods.

4. Alcohol.—In experiment 29 of June 29, 1911, the sperm cells were kept in a series of weak alcohols for 35 minutes. The unfertilized control showed no jelly and no segmentation. The fertilized control was normal. Sperm which had been exposed to the action of 10 per cent. alcohol (made up with sea water) were incapable of producing any effect upon the eggs. 8 per cent. alcohol gave similar results. A few eggs, about 2 per cent., were fertilized by sperm cells which had been kept in 6 per cent. alcohol. Of the 15 eggs which were observed to form jelly, 2 did not segment, one divided only once, and the remainder were motile on the second day. Of the motile forms one half were abnormal in some way upon the fourth day. During the course of the whole experiment this culture was greatly retarded in development. Sperm from the 4 per cent. alcohol fertilized 25 per cent. of the eggs and, with the exception of a few unsegmented eggs, all developed the power of motion upon the second day, though they did not develop so rapidly as did the controls. On the third day at least 3 per cent. were visibly abnormal in body form or in setæ. The fourth day showed 10 per cent. to be abnormal. Some never formed any setæ, some formed weak ones, and some were nearly perfect in this respect. Irregular bunches were often present upon the sides of the segments. The apparent age of the animals in the culture varied greatly. 2 per cent. alcohol did not prove to be so effective, for 60 per cent. of the eggs were fertilized and developed with fewer abnormalities than any of the cases given above.

Other experiments show that sperm cells exposed to weak grades of alchool for a longer time give about the same results as those exposed to higher strengths for shorter periods. Early segmentation stages do not appear to be so much affected as the larval stages, though usually there were at least a few eggs which

failed to segment after jelly formation and maturation or which failed to segment more than once. There was in all cases a strong tendency for the experimental cultures to die earlier, to be less active, and to contain forms which were abnormal in body form or in setæ formation. Permanent trochophores were also common.

In this set seven experiments were completed, involving in the neighborhood of a hundred cultures. Several slightly different methods of procedure were used but the results in all cases were similar to those cited above. An extra control was carried in each experiment. This consisted of eggs and normal sperm to which was added at the time of insemination as much alcohol as was transferred to the experimental cultures along with the sperm used in insemination. In no case did this small quantity of alcohol cause this control to develop in an abnormal manner. This indicates that the abnormalities seen in the experimental cultures were not due to the presence of such minute quantities of alcohol in the egg culture.

5. *Sodium Hydroxide.*—In this group but few experiments were made. The sperm cells were exposed to the influence of different strengths of NaOH for a period of 20 minutes. The solutions used were made up by adding to 10 c.c. of sea water a certain number of drops of 0.75 per cent. NaOH solution. After exposure to one of these mixtures for 20 minutes the sperm cells were used for inseminating fresh eggs. Three controls were carried. The first, eggs only, showed no change. The second, eggs and normal sperm, developed in the usual manner. To the third, similar in composition to the second, was added the same amount of NaOH solution that was carried over to the eggs by the sperm in the experiment 40.4. This likewise developed in normal fashion, thus demonstrating that the trace of alkali necessarily introduced in the process of insemination of the experimental series was without any apparent effect upon early development. In table I. the results of a typical experiment are given.

The early history of the experiment was not followed on account of a lack of time. The observed abnormalities were chiefly in the failure of the trochophores to develop, in which

case irregular bunches appeared on the body which became enlarged and transparent. This appearance was not due to post mortem changes, for the animals sometimes continued to swim about slowly. Many times the setæ failed to appear as has been described in other cases. In the case of 40.5 and 40.6 the smaller abnormality is probably due to the greater mortality, since the abnormal individuals are often not very resistant. The general impression given by this series is similar to that formed from the observations on the alcohol series.

TABLE I.

EXPERIMENT 40. July 21, 1911. All inseminated at 9:30 A.M.

No.	Drops NaOH.	Per Cent. Jelly at 9:55 A.M.	Time of First Cleavage.	Per Cent. Segment.	Later History.
40.1	1	100 –	10:38 A.M.	100 –	Normal.
40.2	2	100 –	10:39 A.M.	90	5 per cent. abnormal in setæ or in having permanent trochophore.
40.3	3	95	10:39 A.M.	90	About like 40.2. More weak setæ.
40.4	4	60	10:43 A.M. and scattering	40	About like 40.3.
40.5	5	40	10:41 A.M. and scattering	15	Development slower. Many die. Living less abnormal than 40.4.
40.6	6	10	11:00 A.M. and scattering	5	Like 40.5 but more abnormal and more die.

6. Hydrochloric Acid.—The effects of acid upon the sperm cells were also observed, the experiments being conducted in the same manner as the alkali series. A number of drops of 9 per cent. HCl were added to 10 c.c. of sea water and the sperm were placed in this solution for 25 minutes. All three of the controls were perfectly normal. Eggs inseminated by the sperm suspension containing one drop of acid often stopped development in late cleavage. Others formed irregularly shaped permanent trochophores. Later over 50 per cent. of the larvæ were abnormal in body form and character of the setæ. The eggs inseminated by the sperm suspension containing two drops of acid frequently failed to cleave. A few permanent two-cell stages were produced. Some of the abnormal larval stages again appeared but not so large a proportion as in the previous case. More than two drops

of acid affected the sperm cells so much that the eggs inseminated by the sperm cells so treated were unchanged or merely formed jelly and polar bodies without segmenting.

An attempt was made with weak NaOH solution to neutralize the acid in which the sperm cells were placed before insemination but this process so often destroyed the life of the sperm cells that it was useless for control purposes. However, the controls used seemed to be sufficient to show that the cause of the altered development does not lie in the presence of the acid in the experimental cultures of eggs.

7. *Cold.*—Freezing the sperm was effected by placing them in sea water in a test tube and imbedding the tube in a mixture of powdered ice and salt. − 8° C. killed the sperm. − 2.2° C. gave a mushy ice in the test tube. The sperm cells were not killed but caused a normal cleavage in the eggs. From 5 to 10 per cent. of the larvæ were abnormal. A very few scattering abnormalities of all the kinds previously mentioned were observed. − 1.6° C. did not seem to affect the sperm cells enough to alter the egg development.

Since low temperatures are very difficult to handle by means of the crude methods employed extensive experiments were not carried out along this line. Refrigeration at a temperature considerably above freezing for longer periods was also used as a possible source of injury to the sperm cells. Since the effects produced are probably due as much to the delay as to the cold these experiments have been mentioned under a previous heading.

B. Upon Arbacia.

In July, 1911, a series of 18 experiments involving about 200 cultures was carried out, using the sexual products of the common sea urchin, *Arbacia punctulata*. Along with some other investigators I had considerable trouble at that particular time in getting control experiments to develop in a normal manner. Accordingly the work was not carried as far as it seemed desirable at that time. Although the results were perfectly convincing to one who could see the living material, the small percentage of abnormality in the controls might cause some to question the value of the published data. I will, however, give a brief survey

of the work since it is in line with the work upon *Nereis* and since some of the features here described have not been previously recorded.

Experiments were carried out by inseminating eggs with sperm which had been subjected to injurious conditions for a longer or shorter time. The injurious agents used were (*a*) sea water concentrated by boiling; (*b*) sea water diluted with distilled water; (*c*) sea water with from one to twelve per cent. of alcohol added; (*d*) sea water slightly acidified with HCl; (*e*) keeping the sperm cells from 6 to 24 hours after removal from the body; and (*f*) combinations of the above.

In a general way these methods of treatment did not give specific effects but produced very similar results, although the intensity of the effects varied as was to be expected. In one case the controls were nearly as abnormal as the experimental cultures. But, taking the experiments as a whole, there can be no doubt as to the great differences to be found between the controls and the experimental series.

The abnormalities produced by inseminating normal eggs with injured sperm may be briefly reviewed. In a few cases in which the sperm cells were exposed to extreme conditions, insemination gave a number of cases of membrane formation without development. Cleavage was found to be extremely irregular in some cases, producing fantastic forms, such as ciliated plates and rows of cells. The more normal cleavage was also found to be irregular but it is difficult to say that it was more irregular than it sometimes is in normal cultures. The development of the experimental series was often found to be retarded, especially after late cleavage. The early cleavage was not affected so much, though occasional indications of a slight retardation were observed. Curiously enough, in some of the experiments using alcohol, the experimental material was observed to be one or two cleavages ahead of the controls in the early segmentation stages, though the difference was soon in the other direction, if any was apparent. Prismatic forms were often observed in the controls when the experimental cultures were chiefly in the blastula stage. The death rate was much higher in all of the experimental series, the whole culture sometimes dying in various

stages from blastula to pluteus before any considerable number of deaths were observed in the controls. A very small number died during cleavage in stages beginning with the undivided egg. Blastulæ whose cavities were more or less filled with cells, similar to the stereoblastulæ so often described by experimental workers, were found in increasingly large percentages as the strength or duration of the injurious agent acting upon the sperm was increased. In some cases over 15 per cent. of the experimental cultures were of this form. They did not appear to develop farther but went to pieces. Evaginate gastrulæ were formed rather uniformly throughout the experimental series and occasionally one or two were found in the controls. The proportion of this type of abnormality did not seem to show so close a correlation with the degree of injury to the sperm as did the stereoblastulæ. Other types of abnormal gastrulæ were very common, this stage seeming to be a difficult one to pass. Some were observed to be much smaller in size than the normal type, perhaps due to a separation of the blastomeres in the early stages. Others were irregular in various ways. The most common type of this irregularity is what I have called in my notes "ragged." These forms do not possess such a clean cut appearance but are covered with minute irregularities and look as though they were going bad. The plutei were variable in the controls, but they were very much more so in the experimental cultures. The arms sometimes failed to form or appeared only as small buds. Usually however the arms were longer and more slender than in the controls. Several cases were observed in which the anal opening did not form. In all cases after the first appearance of motility a much larger number was found in the experimental cultures lying motionless on the bottom of the dish. If disturbed they quickly settled down to their former condition of rest. Some seemed to entirely lack the power of motion although this was not certainly determined.

In general all of the results point toward a great disturbance in the development of all or nearly all parts of the organism. This disturbance seems to be produced by injury to the sperm cells used in insemination. An attempt to see if, in the case of the experiments using alcohol, the disturbance was due to a

possible trace of alcohol carried over with the sperm in the process of insemination, was made by adding alcohol to the control cultures at the time of insemination. The addition in quantities comparable to the amount present in the experimental cultures was without effect. Table II. gives the principal features of a representative experiment, the minor features being omitted.

TABLE II.

EXPERIMENT *A*2. July 4, 1911. Sperm removed at 9:00 A.M. from a male with testes about half soft. Two drops of thick sperm placed in 10 c.c. of each grade of alcohol at 9:15 A.M. and left there 5 hours and 20 minutes. Eggs about 2 per cent. immature. Used immediately after removal. All except *A*2.0 (the uninseminated control) inseminated at 2:35 P.M. Discontinued July 7, 1911.

No.	Per Cent. Alcohol.	Condition at 3:30 P.M., July 4.	Condition at 11:00 A.M., July 5.	Condition at 2:30 P.M., July 5.	Condition A.M., July 7.
*A*2.0	0	Normal.	Normal. No cleavage.	No cleavage. Breaking down.	
*A*2.1	0	2-cell.	Normal gastrulæ.	Late gastrulæ. Trace abnormal.	10 per cent. abnormal.
*A*2.2	2	2-cell.	Gastrulæ 10 per cent. ragged.	Many abnormal late gastrulæ. Ciliated plates seen.	Mostly abnormal.
*A*2.3	3	2-cell.	Gastrulæ 50 per cent. ragged. 1 per cent. stereoblastulæ. A few 2 cells. 1 morula.	As at 11:00 A.M. only more developed.	60 per cent. very abnormal.
*A*2.4	4	2-cell.	40 per cent. abnormal. 1 per cent. stereoblastulæ.	As at 11:00 A.M. but more developed.	80 per cent. abnormal.
*A*2.5	5	2-cell.	Mostly abnormal gastrulæ. 4 per cent. stereoblastulæ.	As at 11:00 A.M. but more developed.	All dead.
*A*2.6	6	No cleavage.	Trace swim. 5 per cent. stereoblastulæ.	As at 11:00 A.M. but more developed.	All dead.
*A*2.7	7	No cleavage.	None swim. 11 per cent. stereoblastulæ.	Dead.	
*A*2.8	8	None fertile.	None fertile.		

V. CYTOLOGICAL STUDY.

A. Normal.

I have found it necessary to go into some of the cytological details which are to be found in the egg of *Nereis* after insemina-

tion with normal sperm. These features have been described previously (Lillie, F. R., '11, '12) but an outline of the cytological changes is necessary for a proper understanding of the observations on the experimental material. My studies have been based upon my control series and upon slides loaned to me by Professor Lillie. The latter are especially fine and show clearly all of the conditions which he has described in his papers. Immediately after a spermatozoön becomes attached to the egg by means of its delicate perforatorium the contents of the coarse alveoli of the cortical layer of the egg begin to pass to the exterior where, in the course of fifteen minutes, they form a thick layer of jelly. A perivitelline space now occupies the position formerly held by the cortical layer of the egg. An entrance cone forms from the egg cytoplasm just beneath the spermatozoön and, about three quarters of an hour after insemination, draws the sperm head, in the form of a thread, into the egg, leaving the tail and middle piece outside. This usually occurs during the late anaphase of the first maturation spindle. The sperm head now begins to grow larger and, retaining its connection with the entrance cone, penetrates the egg protoplasm. The whole complex, as it passes to the yolk free area of the egg, rotates so as to bring the sperm nucleus ahead and nearer to the center of the egg. Meanwhile the sperm aster appears at the pole of the nucleus which is opposite the cone and soon divides, forming an amphiaster. Following the formation of the second polar body, the egg aster fades away and the egg chromosomes swell and form conspicuous vesicles, each one with a distinct chromatic nucleolus, which slowly fuse together to form the egg nucleus. By this time the sperm nucleus has increased in size and presents an appearance much like that of the egg nucleus, near which it has come to lie. The two nuclei now fuse and the asters of the first cleavage spindle, presumably derived from the sperm amphiaster, are seen on opposite sides of the cleavage nucleus. The process of cleavage then ensues.

B. Experimental.

In the experimental material it has proved difficult to establish a definite seriation in the cytological observations, since, as

the observations on the living material indicate, in the experimental cultures different eggs start to develop at different times. This seems, at least in part, to be due to the relatively low activity of the injured sperm cells and to the consequent delay in attachment. Likewise it has been hard to get any satisfactory estimation of percentages of the various stages present in any preservation. The reason for this lies in the fact that in most cases where marked results were obtained, only a small fraction of the eggs were fertilized and too few fertilized eggs were secured from any one series to get counts large enough to be significant in such complex conditions. The losses in sectioning and staining also add to the difficulties. Yet it has been possible to secure the main facts by going over a large number of slides and by comparing the results of different experiments.

Cytological study of the preserved samples from the experimental cultures of *Nereis* eggs shows the following classes of eggs.

(*a*) Eggs not entered by sperm. No segmentation.
  1. Polar bodies formed. Jelly not extruded.
  2. Polar bodies formed. Jelly extruded.

(*b*) Eggs entered by sperm. Segmentation.
  1. First cleavage not completed.
  2. Multipolar mitosis. Maturation incomplete.
  3. Multipolar mitosis. Maturation completed. Polyspermy.
  4. Miscellaneous abnormal eggs.
  5. Apparently normal eggs many of which would doubtless have proved to be defective had they been allowed to develop.

Cytological study reveals nothing as to the cause of the abnormalities occurring after the time of the first cleavage, with the single exception of the above mentioned multipolar mitosis.

(*a* 1) As is indicated by the records made from examination of the living material, by the India ink method, a partial or total lack of jelly formation is one of the striking things which is to be found in nearly all of the experiments. I have never seen this in normally fertilized eggs, either whole or in sections,

although egg sections from early preservations of the experimental series show in some slides as many as 5 per cent. of the maturing eggs with the alveoli of the cortical layer still wholly or partially filled. In most cases an attached sperm cell may be found outside the vitelline membrane. Since it is in an exposed position, not protected by jelly as in other eggs, its absence may be interpreted as due to its accidental removal after preservation. I have not found fertilization cones in sections which show any considerable amount of material still present in the cortical alveoli. Maturation goes on in an apparently normal fashion (Fig. 1) though probably much slower than usual since the later maturation stages of this type are not seen until the normal maturation stages have been completed for some time. I have found most of the principal maturation stages, from the breaking down of the germinal vesicle to the formation of the vesicles of the female nucleus, and, with the exception of the cortical layer, they seem to be like the normal stages. The polar bodies either come to lie imbedded in the cortical layer, or are pushed entirely through it to the outside (Fig. 2). After the formation of the second polar body the chromosomes, in the usual manner, rapidly form a number of large chromosomal vesicles with the haploid number of chromatic nucleoli. But no indication of the sperm is to be seen within the egg. No trace of cytoplasmic radiations is ever seen within the egg after the maturation phenomena are completed and the sperm nucleus is not to be found unless the remnants of the sperm cell are still attached to the outside of the vitelline membrane. The chromosomal vesicles, not being able to fuse with the sperm nucleus, may now behave in one of several ways. They may scatter at random through the cytoplasm or they may retain their position beneath the polar bodies, in which case they usually form a more or less hazy, poorly staining mass of irregular shape. In a few cases I have found about a dozen separate or partially fused vesicles containing in all about 26 chromatic nucleoli. At first I thought that this was a case in which the sperm had entered. But the entire absence of cytoplasmic radiations, the irregular arrangement of the vesicles, and the determination of approximately the haploid number of vesicles indicates that we are dealing with a case of multiplica-

tion of the chromatic nucleoli without true fertilization. In some cases the chromosomal vesicles seem to dissolve in the cytoplasm, leaving behind several small chromatic nucleoli.

To sum up, the sperm attaches itself to the egg which slowly undergoes maturation but does not form jelly or segment. The sperm does not enter the egg and no fertilization cone is formed. After the formation of the second polar body, the chromosomal vesicles of the egg nucleus form and, failing to unite with a sperm nucleus, undergo degenerative changes. The spermatozoön thus gives the egg a stimulus which induces only the formation of the polar bodies. In such cases the maturation process goes on in a normal manner with the exception of a retardation in the rate. This retardation is probably caused by the retention of the jelly-forming materials in the cortical layer of the egg, which would tend to cut down the rate of exchange with the external medium.

It is possible that we are dealing in this case with eggs which require a rather high degree of stimulation in order to produce jelly. Observation on unfertilized eggs shows that there is a considerable degree of variation in the amount of stimulus required to produce jelly formation. It may be that the injured sperm cells are incapable of giving so great a stimulus for jelly formation as do the normal ones and that some of the eggs cannot respond to the slight stimulus given, by emptying their cortical alveoli, although they may be able to form polar bodies. Whether the cause lies in the egg or in the sperm it is necessary, since these eggs develop normally if fertilized with uninjured sperm, to assume that the injury to the developmental process is produced by the action of external influences upon the sperm cell before the time of insemination. It is also clear that in the case of *Nereis* eggs maturation may take place without jelly formation.

(*a* 2) In a much larger number of cases the eggs go a step farther than those described above. The living material shows eggs which undergo both maturation and jelly formation but which never segment. Although, in the earlier stages, a sperm cell may be seen to be attached to the vitelline membrane no fertilization cone is present. Examination of the sections of such eggs

completely confirms the observations made upon the living material. The sperm cell becomes attached to the vitelline membrane, the processes of maturation and jelly formation follow in a normal manner, and the animal pole of the egg becomes free from yolk. But no fertilization cone is formed and no attachment granules appear at the end of the perforatorium (Fig. 4). The egg protoplasm just beneath the attached sperm shows no more change than that at any other part of the egg. The perforatorium, which in cases of normal attachment becomes wider and more easily seen (Fig. 5), remains as a very delicate process which cannot be followed beyond the vitelline membrane. Large granules, which stain deeply in the iron hæmatoxylin, soon appear in large numbers in the peripheral parts of the egg and also around the egg nucleus. In later stages the sperm head becomes larger and somewhat less dense and often disappears entirely. This is presumably due to secretions which are thrown off from the egg, as has been demonstrated by F. R. Lillie ('12).

No evidence of the presence of a sperm within the egg can be found. At the close of the second maturation period the egg chromosomal vesicles begin to swell and may unite with each other to a greater or less extent (Fig. 3). Cytoplasmic parts of the mitotic figure never appear although there are sometimes traces of chromosome formation within the vesicles in the earlier stages (Fig. 6). In some cases the vesicles separate and scatter through the cytoplasm where they may dissolve and leave behind a number of deeply staining chromatic nucleoli. More often the vesicles remain in place and break down into a mass of poorly staining debris (Fig. 7) in which, in the earlier stages, chromosome-like forms may be visible. Two or three hours after insemination the cytoplasm frequently begins to bud off small pieces filled with deeply staining granules. This usually appears first near the equator. Later the whole cell may break up into parts which more or less resemble blastomeres, although close examination in the living state reveals their nature. In sections it is easily seen that the parts do not possess nuclei.

It is clear that the injured sperm has given the first stimulus of fertilization. But, although the initial stages have been produced, the effects necessary for further development have not

been called forth within the egg. The achromatic parts of the mitotic figure are entirely absent although there are indications that the chromosomes may undergo a part of the changes preliminary to first cleavage The effects produced by the attachment of the sperm seem to be nearly the same as those secured by centrifuging the eggs or by giving them any mechanical stimulus (Lillie, '11).

It is of interest to find that a few of the eggs of the type described above show the presence of two or more sperm cells attached to the vitelline membrane. This has been found in several cultures in which many eggs showed no evidence of having come in contact with a sperm cell. Apparently the weakened sperm cells are not able to promptly call forth in the egg the reaction which prevents the attachment of more than one sperm. The partial jelly formation which was observed in the living material is often associated with the failure of the sperm to enter the egg. However cases have been seen in which the sperm entered and caused first cleavage to take place. The farther history is not known but, since preservations made from somewhat later stages show no traces of such eggs, it is probable that the cortical alveoli are soon emptied.

(*b* 1) A very interesting observation upon the living material has been recorded above. In many experiments a few eggs seemed to have nearly completed the process of first cleavage when the cleavage plane ceased to advance and gradually faded away. Cytological examination fails to pick out these eggs until the early telophase stages of first cleavage. The earlier processes seem to go on in a normal manner. In most cases the chromosomal vesicles fail to unite properly and the plane of cleavage fades away. Usually it leaves behind a change in the arrangement of the yolk granules and its position is occupied by a somewhat lighter staining band in which small granules are less abundant. Parts of the sharp line which marks the completed cleavage plane in sections may be left behind, especially those parts near the animal pole of the egg (Fig. 8). The chromosomal vesicles sometimes scatter widely and partially dissolve and sometimes they remain near each other. Very often the region near the vesicles becomes filled with deeply stainirg granules. In one case

a cell was found in the late prophase of second cleavage, though without a first cleavage plane. In this case the nuclei have been able to divide again though the cytoplasmic division was never completed. In most cases the cells which fail to finish the first cleavage never go any farther.

(*b* 2) A very few eggs were found in each of the experiments which exhibited the above described attempted cleavage, in which multipolar spindles were found. Unfortunately my preserved material contains very few of these eggs and the sections of some of them were lost or badly broken in the process of preparation.

There are two different kinds of eggs which show this condition. One kind has formed two polar bodies. There are very few of these eggs which are in a condition good enough to work upon. Although the cultures from which the eggs were taken were more than 75 per cent. unfertilized these eggs seem to show evidences of polyspermy. This is borne out by the fact that, in a few cases, two sperm cells were seen to be attached to a single living egg in cultures similar to those from which these eggs were taken. Although it is conceivable that multipolar figures might arise from causes other than polyspermy, it is very unlikely. I regard the few observations of polyspermy in the living cultures as good evidence that the few cases under consideration are due to the entrance of more than one sperm cell. The observations mentioned under (*a* 2) above also point in this direction.

(*b* 3) The other kind of multipolar figures is found in eggs which show defective maturation. In one case an egg was found in which the first polar body had formed. Just beneath it lay 14 chromosomal vesicles and in the center of the section about 20 vesicles lay near a strong cytoplasmic radiation. The presence of the radiation probably indicates that a sperm nucleus has fused with the egg nucleus, although the number of vesicles which can be made out is below the diploid. Another sperm cell lies in contact with the vitelline membrane, probably attached to it. Although this is not a case of multipolar mitosis in itself it is possible that the 14 vesicles near the animal pole, which represent the second polar body, may enter into the cleavage process and aid in the production of multipolar figures. One or

two cases of such figures in eggs with but one polar body have been noted in late cleavage stages. Other eggs may fail entirely to produce polar bodies. In these eggs from six to nine poles form and the spindles are often very complicated and produce an irregular distribution of the chromosomes, which, in the earlier figures, take the form which is found on the second maturation spindles. It has been shown (Lillie, F. R., '11) that the formation of the first polar body may be prevented by the use of the centrifuge. In such cases multipolar figures of various kinds are formed. Clearly my case is due to the suppression of the polar bodies. I have not seen similar cases in sections of normal series though, in such small numbers as are found in the experimental series, it is possible that they may have been overlooked.

(*b* 4) A few scattering abnormalities have also been found but not in sufficient numbers to be of any significance. Two cases in which the sperm and egg nuclei fused together and failed to develop farther have been seen. Two cases of prophase figures from second cleavage were found in which the spindles lay at right angles to each other in different planes. Other abnormal conditions were also found. All of these cases are open to the interpretation that the egg was abnormal before fertilization and are not worth description.

Careful search was made for eggs showing abnormalities at the period of entrance of the sperm. It seemed likely that the stages of fusion of the egg and sperm nuclei would contain evidence of abnormality but close examination failed to show anything of the kind, except in the case of the two eggs already mentioned. Since a kind of parthenogenetic cleavage might be produced by the partial action of the sperm cell, search was made for eggs exhibiting the haploid number of chromosomes in the first cleavage. But in all cases the number was clearly diploid.

VI. Discussion.

A. The Production of Defectives.—For a long time it has been assumed by many that the offspring of parents who habitually take alcohol or other drugs or who work in lead are very apt to be in some way weak or defective. Some of the unfortunate

conditions in the offspring which different men have associated with parental alcoholism are insanity, epilepsy, feeble-mindedness, cretinism, macrocephaly, lack of self-control resulting in criminality or in over-indulgence in alcoholic drinks, malformations, retarded development, poor health, and the like. Medical men often state that there is every indication that a great number of abortions, stillbirths, and births of defectives are definitely connected with times of conception corresponding to periods of drunkenness, or drug craze, or of working in lead. So much has been written on both sides of the question, especially with reference to alcohol, that it is impracticable to attempt to give any exhaustive review of the literature. Unfortunately, also, so much of the writing is the product of prejudice and of a desire to establish some point, regardless of the evidence, that it is difficult to separate the wheat from the chaff.

There are three theories to be mentioned in connection with the relation of alcoholism to the production of defectives. (1) Since the excessive use of alcohol unquestionably produces undesirable effects upon the body of the consumer, many believe that the offspring of an alcoholic parent may in some way inherit certain weaknesses thus produced. But it seems that at present we have no reason to believe that *specific* somatic alterations of the parent, produced by drugs or otherwise, are inherited by the offspring. Consequently any such theory may be rejected at once.

(2) Some writers go so far as to assert that there is no direct *causal* connection between parental alcoholism and defectiveness in the offspring. They maintain that alcoholism, insanity, feeblemindedness, epilepsy, etc., are often merely different forms of expression of a single weakness. In many cases, as stated by W. Branthwaite, H. M. Inspector under the Inebriates' Act ('08), alcoholism is undoubtedly caused by some weakness which is present in the family. There seems to be sufficient evidence to show that this statement is true. Yet we cannot accept the theory that this hereditary weakness entirely explains the relations under discussion.

(3) Very few writers have even ventured to suggest that the defective conditions appearing in the offspring of alcoholics

may be due in part to the direct action of the alcohol upon the germ-cells; in case the father alone be alcoholic that his spermatozoa may be so affected as to induce abnormal development of the ovum fertilized by him. Until recently there has been little evidence to show that such a theory is tenable and more evidence is desirable. The data presented in this paper demonstrate that the sperm cells of *Nereis* and *Arbacia* may be so affected by alcohol and by other methods of treatment, that their union with normal eggs will produce an abnormal development. As is shown below, there is evidence of a similar nature in other forms.

In man the conditions are so complex that they are hard to analyze, especially since we may not resort to direct experiment. Alcohol poisoning may take place in utero, during the nursing period, or even later. Or it may be that the same depressing conditions which caused the parents to drink may react similarly upon the offspring. Many other difficulties also confront the investigator who seeks to solve the problem by the methods of the past. It seems clear that such methods will never give the solution. Definite biological experiment upon the lower animals must form the chief basis for any conclusions which we may make in the future. With this thought in mind the present series of experiments was undertaken in the hope that some definite evidence might be presented upon the subject.

Although there may sometimes be a suspicion that the data relating to man have been selected with a view of establishing a point rather than seeking the truth, it is worth while to recall a few cases because of their interest in the present connection. Several European investigators (*e. g.*, Schweighofer and Bezzola) are frequently quoted in the literature as having found that there is a definite relation between the time of the greatest number of stillbirths, abortions, and births of mental defectives, and the great feast seasons, during which much alcohol is consumed. From a statistical standpoint such statements have been severely criticized, especially by Pearson and Elderton ('10). There is also little evidence to show that the effect of the alcohol was not exerted upon the developing embryo. Since the effect of the alcohol taken by the mother may be either upon

the egg or upon the developing organism, the most desirable evidence comes from cases in which the male germ cells alone are exposed to the supposedly unfavorable conditions.

Saleeby ('11) quotes Galton as having given the three following cases. A man who had normal children became a drunkard and his later children were all imbeciles. A healthy woman who had by a drunken husband five sickly children who died in infancy, later married a healthy man and produced normal children. A man with two healthy children acquired the cocaine habit and engendered two idiots.

Schweighofer gives a case of a normal woman who had three normal children by a sound man. She later married a drunkard. Of the three children from this union one had infantilism, one was a drunkard, and one was a degenerate. In a third marriage she again bore healthy children.

Paul ('60) tells of the children of lead workers. From 32 pregnancies, the father alone being exposed to the lead poisoning, there resulted 12 abortions, stillbirths, and premature labors, and 20 living births. Of the living 8 died during the first year, 4 during the second, and 5 during the third. Paul states that the influence of the lead is as real as in the cases where the mother is exposed, though perhaps the effects produced are not so great.

The observations quoted above are among the best on record. Many other similar observations can be collected by anyone who thinks it worth the time. A rather full bibliography is given by Hoppe ('12). In the light of recent work these facts are very interesting. Although in some cases the remarriage serves as a control, the lack of data concerning the previous family histories is a defect sufficiently serious to warrant us in questioning any conclusions which may be drawn from these data alone. So far as I have searched there are no observations upon man which meet with rigorous scientific requirements.

Elderton and Pearson ('10) conclude from the statistical study of English school children that the offspring of alcoholic parents are slightly brighter, heavier, and less diseased than those of sober parents and that epilepsy and tuberculosis are of no more frequent occurrence than among the children of non-alcoholics.

They also record a higher mortality among the children of alcoholics and conclude that the more resistant survive. The publication of this paper has been followed by widespread criticism. The details of the individual cases are not given. We do not know what may be included under the terms "alcoholism" and "intemperate." We should know the condition of the children who were not in school, due perhaps to lack of ability or to ill health, the relation of the time of conception to periods of drunkenness, and whether the mother, the father, or both, were alcoholics. In view of the lack of so much desirable evidence and of the heterogeneity of the materials investigated it seems that the question as to the general applicability of the conclusions reached is at least an open one, the more so since the experimental evidence seems to be directly opposed in most cases. Additional light is given by the researches of Nicloux ('00) who proves that alcohol may reach the ovaries and testes of mammals and that these organs take up considerable quantities of the drug. Alcohol is also present in the seminal fluid very shortly after it is taken into the stomach. Accordingly it seems to be possible that the sperm cells may be injured. Bertholet's work ('09) in a sense confirms that of Nicloux, since he finds that testicular atrophy is common among alcoholics. His observations lead one to think that sterility should be much more common than it is among drunkards.

There are a number of published observations upon the lower animals. Mairet and Combemal ('88) foumd that a dog treated with absinthe for 8 months and paired with a normal female gave 12 young. 2 were born dead and the others all died within 11 weeks after birth. The small numbers and the lack of adequate control make this experiment indecisive.

Bardeen ('07) found that toad eggs, when fertilized by sperm cells which had been previously exposed to the X rays, developed abnormally. Since the question involved is wider than the alcohol question these results are significant.

O. Hertwig ('10, '11) and G. Hertwig ('12) obtained similar results by the exposure of the sperm cells of various animals to radium and also by injecting methylene blue into the dorsal lymph sac of the male frog some days before the sperm cells were used to

fertilize normal eggs. In this work it was found that the stronger action upon the germ cells produced earlier and more profound alterations in the offspring.

Stockard ('12) furnishes a set of well-planned experiments upon guinea pigs which, when tested, produced normal offspring. The males were intoxicated by inhaling alcoholic fumes. As a result of 24 matings of alcoholic males with normal females he reports no results or early abortions in 14 cases, 5 stillborn litters, and 5 living litters containing 12 young. Of these, 7 died shortly after birth. The remaining 5 "are unusually small and very shy and excitable animals." The parents remained in good health throughout the experiment. Although the number of matings is rather small there is a clear indication that the action of the alcohol upon the male germ cells is the cause of abnormal results. Considered in connection with the work of Bertholet and of Nicloux this is very strong evidence.

The experiments of Nice ('12) do not seem to entirely agree with the experiments just quoted. His mice, both male and female, were fed on milk and crackers, to which 2 c.c. of 35 per cent. alcohol was added daily. They were also furnished with drink in the form of 35 per cent. alcohol. The fecundity of the mice was greater than that of the control series, though the mortality was 11.1 per cent. in the offspring in the experimental series and zero in the controls. None of the young were deformed. Nicotin, caffein, and tobacco fumes gave similar results. In the absence of farther experimental data it seems fair to assume that the germ cells of the mice are in some way less susceptible to these drugs, though the comparatively high mortality indicates that there was an effect.

Gager ('08) subjected pollen grains to the action of radium and secured a marked change in the plant resulting from pollination of a normal plant. Some of the effects persisted through several generations.

My own experiments upon *Nereis* and *Arbacia* demonstrate that injury of the sperm cells by several methods may produce a series of abnormalities which may appear in various stages from the time of insemination up to late larval stages. It is not known whether, if the animals were kept under favorable condi-

tions for life and growth, other abnormalities would occur in later stages, but since experimental cultures which are apparently normal in the earlier stages frequently develop abnormalities in the larval condition, it is very probable that other changes would come to the surface in still later stages. In the *Nereis* experiments there can be no doubt as to the cause of the abnormalities observed. The controls, using the sexual products from the same animals, exclude the possibility of abnormalities being present in the lines used. The very large numbers handled demonstrate that the results are not due to the chance outcropping of hidden defects. The series composed of *Arbacia* material, if standing by itself, might be questioned. But the general type of results is so similar to that obtained by Bardeen, the Hertwigs, Stockard, and myself on other forms that there can be no question as to their applicability.

The lack of specificity in the action of the agents used is remarkable. At first thought it seems strange that acid, alkali, alcohol, heat, delay, and other means should produce similar results. It is clear that the action must be much the same in all the cases which I have recorded as well as in those recorded by the authors cited. At present we can neither assign a definite reason for the lack of specificity in the results nor tell how the abnormalities are produced. I can only suggest that the sperm cells are affected in such a manner that their vitality is lessened, or in other words, their rate of metabolism is lowered. It is known that the rate of metabolism in the normally fertilized egg rises rapidly after the time of fertilization and continues to rise for some time. Child ('11) has shown that in the regulation of pieces of *Planaria* the type of structure formed may be definitely controlled through changes in the rate of metabolism produced by means of low temperature, anæsthetics, carbon dioxide, etc. He finds that, in a general way, regions of normally low rate, such as posterior regions, are most affected. Any particular process of morphogenesis seems to require a certain minimal rate of metabolism for its normal completion. It is possible that we are dealing with a similar case. The injured sperm cell may be unable in some cases to give to the egg a sufficient stimulus to raise the rate of metabolism to a point

where it is able to draw in the sperm head. In other cases the sperm head enters and the rate may be increased greatly but not enough to cause the normal completion of many processes. In some cases the rate may be so low that cleavage cannot take place in a normal manner. Since nearly all of the forms investigated show abnormalities at the period of gastrulation it may be that the minimal rate necessary for normal gastrulation is often not reached by the eggs in the experimental cultures. In *Nereis* at the period of elongation following the trochophore stage, there is again a production of abnormalities in the posterior region. Although the evidence is very fragmentary at present it seems that the lack of specificity in these experiments is perhaps capable of explanation upon the basis of lowered rate of metabolism produced by injury to the sperm. Farther research will be necessary before we can do much more than venture a guess as to the solution of the problem.

We are safe in concluding from the observations upon man and the higher mammals and from the experimental work upon mammals, amphibians, annelids, echinoderms, and the higher plants, that it is possible to injure the male germ cells by the application of external forces so as to produce a change in the next generation at least. We cannot say just how long this change may persist. The work of Gager upon plants indicates that changes so produced may persist through several generations. Tower's work upon the female germ cells of *Leptinotarsa* indicates that the changes may persist or may gradually fade away. In all probability the results obtained by the methods which I have used will prove to be for the most part transitory, although there is no reason to believe that some of them may not persist.

There is, then, good reason to believe that some drugs, such as alcohol and cocaine, are a detriment, not only to the consumer but also under certain conditions to his offspring. Since alcohol appears in the seminal fluid very shortly after being taken into the stomach, there is good reason to believe that a man, intoxicated for the first time, even, may beget offspring which will be in some degree defective. It is also possible, though not demonstrated, that, in addition to drugs taken voluntarily into the system, the products of abnormal metabolism may exercise a

similar influence. It is even conceivable that nervous states may be able through alteration of somatic metabolism, to affect the germ cells. Many other possible causes of abnormality might be mentioned. But speculation is useless at this time and experimentation is needed. A large field for investigation is opened up and the results of experiments in this field cannot fail to be of interest to the student of eugenics. The sociological application of the observations here recorded is sufficiently obvious.

B. Fertilization.—The results of these experiments are completely in accord with those given by F. R. Lillie ('11 and '12). So far as my observations extend they are practically identical with those given by Lillie. He finds that if the attached sperm cell is removed by centrifuging, the processes of jelly formation and maturation go on in a normal manner, though cleavage does not result. My experiments demonstrate that if the sperm is injured so much that it fails to enter the egg, essentially the same results are secured. This fact again supports the view expressed by Loeb ('09), Lillie ('11 and '12) and Bataillon ('12) to the effect that at least two factors are involved in the process of fertilization. The first is concerned with membrane formation and, in itself alone, is insufficient. Certain cytoplasmic changes such as the rearrangement of the yolk granules are also produced by the initial stimulus. Since slight stimuli cause jelly formation and maturation, pricking as in Bataillon's experiments is probably sufficient to produce these changes. Certainly it is difficult in *Nereis* to see any action of the sperm beyond the attachment of the perforatorium, which is responsible for the early changes in the egg. In *Nereis* it is evident that maturation may take place in the absence of membrane formation if the stimulus given by the sperm is sufficiently light. The second factor has to do with the internal stimulus. It is a difficult matter to determine at just what stage fertilization is complete. In all cases which I have observed, the formation of a fertilization cone is succeeded by the entrance of the sperm head and by the formation of the first cleavage spindle. The internal stimulus is not yet completed, even in the case of fertilization by a normal sperm cell at the time when the fertilization cone is formed. This is shown in

Lillie's experiments by the removal of the sperm cell at this stage. Since the formation of attachment granules and a fertilization cone is not in itself necessary for membrane formation, maturation and other visible cytoplasmic changes, as is shown by my experiments, and since their presence without the sperm head, does not lead to a greater visible change than does their absence, there is good reason to believe that they function solely in the attachment, penetration, and revolution of the sperm head. After these functions have been performed they cannot be traced much farther. They are probably dedifferentiated and behave as ordinary cytoplasm. I am inclined to think that in *Nereis* the mere penetration of the egg cytoplasm by the sperm head is insufficient although the observations on this point are not entirely satisfactory. The experiments of Ziegler ('98) and Wilson ('03) support this view. Ziegler succeeded in producing a constriction in the egg of the sea urchin in such a way that it separated the egg nucleus from the sperm nucleus. The part containing the sperm nucleus segmented. The remainder failed to segment, but gave indication that the presence of the sperm nucleus was not without effect, by dissolving and reappearing several times.

Wilson cut the eggs of *Cerebratulus* in two, shortly after the penetration of the sperm cell. When the cut separated the two nuclei the part containing the sperm nucleus segmented and the other part formed polar bodies but refused to cleave.

Even in cases where the germ nuclei of *Nereis* copulate there is not necessarily a complete stimulus to development. F. R. Lillie thinks that the partial sperm nuclei produced by centrifuging at the proper time do not cause normal cleavage. Sometimes a partial cleavage results and sometimes cleavage ceases in the two-cell stage. In my experiments many eggs either attempted to cleave and failed or stopped after cleaving once. This indicates that the mere presence of the germ nuclei is insufficient as an internal stimulus for development. The normal interchange between the nucleus and cytoplasm is not necessarily brought about. Although the nucleus of the sperm succeeds in producing aster formation yet the rate of metabolism is so low that normal cleavage cannot take place or can take place

only once or twice. Although we have no direct evidence as to the comparative rates of metabolism of eggs fertilized with normal sperm and those fertilized with injured sperm it seems possible that a difference exists. The retardation in development, the higher mortality in the later stages, and the differences in phototropic response indicate that this is likely.

VII. Summary.

1. *Nereis* eggs, inseminated with sperm cells which have been injured by one of several methods, may fail to develop in a normal manner.

2. In some cases the egg does not form a fertilization cone, attachment granules are lacking and the sperm head is not drawn into the egg but remains outside attached to the vitelline membrane. There are two classes of such eggs, neither of which segment, viz.: (*a*) Those which slowly undergo maturation without forming jelly. (*b*) Those which form both jelly and polar bodies.

3. Those eggs in which the sperm head enters, form the first cleavage spindle in an apparently normal fashion but may fail to complete the division of the cytoplasm. Those which complete this division may develop abnormalities in later stages.

4. The eggs of *Arbacia* exhibit a similar series of abnormalities when fertilized by weakened sperm cells.

5. There is no indication of specificity in the action of the agents used in injuring the sperm cells.

6. These experiments demonstrate that eggs fertilized with sperm cells injured by alcohol and by other means may produce abnormal forms. Taken in connection with the demonstration by others that the germ cells of mammals may be exposed to injurious conditions this has an important bearing upon the relation of alcoholism to the production of defectives.

7. There seem to be at least two factors involved in the process of fertilization. The one has to do with membrane formation and certain other changes, the other with the internal stimulus.

8. In *Nereis* the presence of the two germ nuclei within the egg is not necessarily sufficient as an internal stimulus for normal development.

I am indebted to Prof. Frank R. Lillie for suggesting this line of work and for his many kindnesses during the progress of the work.

LITERATURE CITED.

Bardeen, C. R.

'07 Abnormal Development of Toad Ova Fertilized by Spermatozoa Exposed to Roentgen Rays. Jour. Exp. Zoöl., Vol. 4.

Bataillon, E.

'12 La parthénogénèse des amphibiens et la " fecondation chimique " de Loeb. Ann. des. Sci. Nat. Zoo., Ser. 9, Tom. 16.

Bertholet, E.

'09 Ueber Atrophie der Hoden bei chronischen Alcoholismus. Centralbl. f. allgem. Path., Bd. 20.

Branthwaite, W.

'08 Inebriety, its Causation and Control. Brit. Jour. Inebriety, Jan., 1908.

Child, C. M.

'11 Experimental Control of Morphogenesis in the Regulation of Planaria. Biol. Bull., Vol. 20.

'12 The Process of Reproduction in Organisms. Biol. Bull., Vol. 23.

Elderton, E., and Pearson, K.

'10 A First Study of the Influence of Parental Alcoholism on the Physique and Ability of the Offspring. Eug. Lab. Mem., X., Dulan, London.

Gager, C. S.

'08 Effects of the Ray of Radium in Plants. Mem. New York Bot. Garden, IV.

Hertwig, G.

'12 Das Schicksal des mit Radium bestrahlten Spermachromatins im Seeigeli. Eine experimentell-cytologische Untersuchung. Arch. f. mik. Anat., Bd. 79.

Hertwig, O.

'10 Die Radiumstrahlung in ihrer Wirkung auf die Entwicklung tierischer Eier. Mitteilung vom 15. Juli, 1909. Sitzungsber. der Königl. Preuss. Akad. d. Wissensch., XI.

'10 Neue Untersuchungen über die Wirkung der Radiumstrahlung auf die Entwicklung tierischer Eier. Mitteilung vom 28. Juli, 1910. Sitzungsber. der Königl. Preuss. Akad. d. Wissensch., XXXIX.

'11 Mesothoriumversuche an tierischen Keimzellen, ein experimenteller Beweis für die Idioplasmanatur der Kernsubstanzen. Mitteilung vom 6. Juli, 1911. Sitzungsber. d. Königl. Preuss. Akad. d. Wiss., XL.

'11 Die Radiumkrankheit tierischer Keimzellen. Ein Beitrag zur experimentellen Zeugungs- und Vererbungslehre. Bonn. Fr. Cohen.

Hoppe, H.

'12 Die Tatsachen ueber den Alkohol. Ed. 4. Munich.

Just, E. E.

'12 The Relation of the First Cleavage Plane to the Entrance Point of the Sperm. Biol. Bull., Vol. 22.

Lillie, F. R.

'11 Studies of Fertilization in *Nereis*. I. and II. Jour. Morph., Vol. 22.

'12 Studies of Fertilization in *Nereis*. III. and IV. Jour. Exp. Zoöl., Vol. 12.

'12 The Production of Sperm Iso-agglutinins by Ova. Science, N. S., 36.

Lillie, F. R., and Just, E. E.

'13 Breeding Habits of the *Heteronereis* Form of *Nereis limbata* at Woods Hole, Mass. Biol. Bull., Vol. 24.

Loeb, J.

'09 Die chemische Entwickelungserregung des tierischen Eies. Berlin, Julius Springer.

Mairet et Combemale.

'88 Influence degeneration de l'alcool sur la descendance. Comptes rend. hébd. des Sci. de l'acad. des Sci., Tom. 106.

Nice, L. B.

'12 Comparative Studies on the Effects of Alcohol, Nicotine, Tobacco Smoke, and Caffein on White Mice. Jour. Exp. Zoöl., Vol. 12.

Nicloux, M.

'00 Passage d'alcool ingéré dans quelques glandes et sécrétions génitales. Comptes rend. Soc. de. Biol., Tom. 52.

Paul, C.

'60 Étude sur l'intoxication lente par les preperations de plomb, de son influence sur le produit de la conception. Arch. gen. de. med., Tom. 15.

Pearson, K., and Elderton, E.

'10 A Second Study of the Influence of Parental Alcoholism on the Physique and Ability of the Offspring. A Reply to Medical Critics of the First Memoir. Eug. Lab. Mem., XIII., Dulan, London.

Saleeby, C. W.

'11 Parenthood and Race Culture. Moffat, Yard & Co., New York.

Stockard, C. R.

'12 An Experimental Study of Racial Degeneration in Mammals Treated with Alcohol. Arch. Int. Med., Vol. 10.

Wilson, E. B.

'92 The Cell-lineage of Nereis. Jour. Morph., Vol. 6.

'03 Experiments on Cleavage and Localization in the Nemertine Egg. Arch. f. Entw'mech., Bd. 16.

Ziegler, H. E.

'98 Experimentelle Studien über die Zelltheilung, II. Arch. f. Entw'mech., Bd. 6.

EXPLANATION OF PLATES.

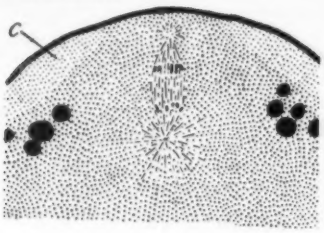
PLATE I.

All figures were drawn with the camera lucida with Leitz apochromat 2 mm. oil immersion objective, and Zeiss No. 6 compensating ocular. *c*, cortical layer.

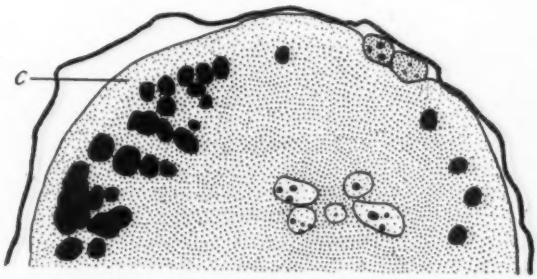
FIG. 1. Egg fixed 1 hour and 20 minutes after insemination with sperm which was injured by heating to 43–44° C. for 8 minutes. No jelly has formed. Peripheral alveoli still intact. Anaphase of first maturation.

FIG. 2. Reconstruction of 2 sections of an egg from same culture as Fig. 1, fixed 1 hour and 50 minutes after insemination. Both polar bodies have been formed. Peripheral alveoli still unemptied. Egg nucleus in form of scattered vesicles, only part of which are seen in this figure. In all, 14 vesicles and about 28 nucleoli are indicated. Sperm cell (in another section) is still attached to vitelline membrane.

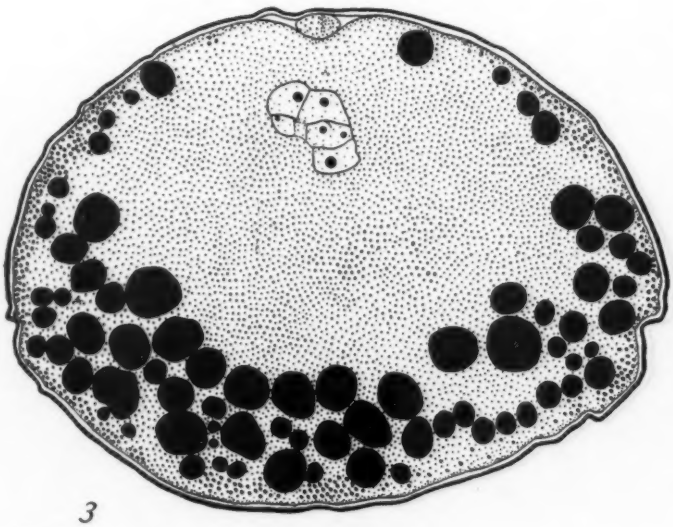
FIG. 3. Egg fixed 1 hour and 15 minutes after insemination with sperm which was injured by heating to 43–44° C. for 17 minutes. Jelly was formed and both polar bodies are present. Second polar body shown. Egg nucleus in form of vesicles, 13 of which are indicated in the various sections. Sperm cell (in another section) is still external. Strongly staining granules around periphery of egg.



1



2



3

NEIL S. DUNGAY.

PLATE II.

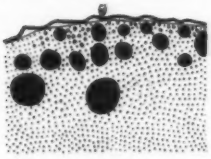
FIG. 4. Egg, from same culture as Fig. 3, fixed 1 hour and 5 minutes after insemination. Sperm attached to vitelline membrane, no attachment granules, and no fertilization cone. Perforatorium still delicate. Jelly and both polar bodies have formed. Vesicles of egg nucleus as in Fig. 6.

FIG. 5. Egg from control culture 43 minutes after insemination, showing fertilization cone, attachment granules, and sperm with thickened perforatorium.

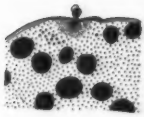
FIG. 6. Egg from same culture as Fig. 3, fixed 1 hour and 27 minutes after insemination. Jelly and both polar bodies have formed. Sperm, in another section, attached to vitelline membrane. Vesicles of egg nucleus showing chromosome within.

FIG. 7. Egg from same culture as Fig. 3, fixed 1 hour and 36 minutes after insemination. Jelly and both polar bodies formed. Egg vesicles degenerating. Sperm (in another section) attached to vitelline membrane.

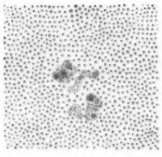
FIG. 8. Egg fixed 2 hours and 17 minutes after insemination with sperm removed from male and kept at room temperature for 21 hours. This egg has not succeeded in completing the division of the cytoplasm and the chromosomal vesicles, about 28 on each side, are scattering. Strongly staining granules around the chromosomal vesicles.



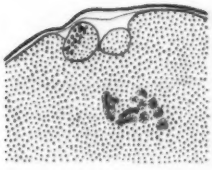
4



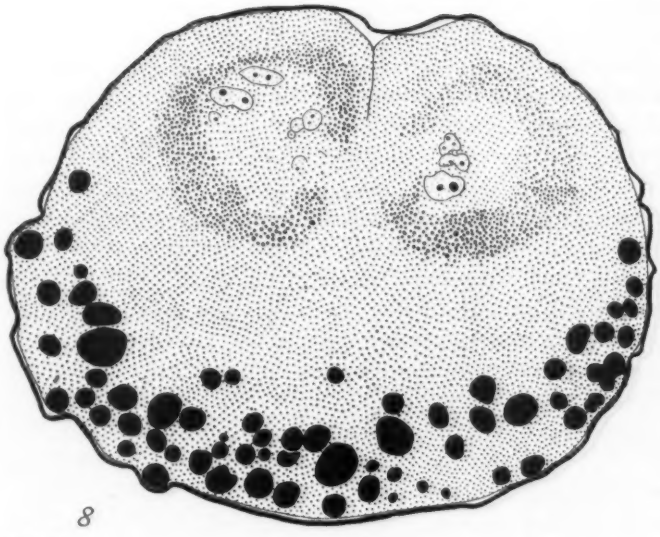
5



7



6



8

NEIL S. DUNGAY.

OBSERVATIONS ON LIVING SOLENOMYA
(VELUM AND BOREALIS).

EDWARD S. MORSE.

The genus *Solenomya*, represented by a few species, is widely distributed throughout the world. It has been found on the east and west coast of North America, in West Africa, the Mediterranean, the Canaries, Australia and New Zealand. The few species agree in almost every detail but vary greatly in size. Our two species, *S. velum* and *S. borealis*, are one inch and three inches long respectively. *S. bartschi* from Manila is eight and one half inches in length. The species vary greatly in the proportional number of individuals. *S. velum* is at times thrown up by thousands on our beaches, *S. borealis* is rarely found. *S. grandis*, which departs more widely from the type than the others, is known by two specimens and a few fragments, while the gigantic *S. bartschi* is represented by a single specimen. The various species have without exception a long semi-cylindrical shell, rounded at both ends, a long and almost straight hinge margin. They all have radiating ribs with an apparent interspace in the series in which however a faint rib may be detected. They all have a highly polished periosteum which runs far beyond the border of the true limy shell, and to which the mantle with its curious system of muscles is closely adherent. The radiating ribs are continued through the overlapping periosteum by a marked thinning of the substance and Professor Drew discovered that when the periosteum folds inward as the valves close these thin areas fold in a plaited manner. In the dry state these interspaces often split and the substance being very brittle it is hard to preserve unbroken in cabinet specimens. The various species vary from a light yellowish brown to a dark brown and even a tar color. The shell within is grayish blue or lead color in *S. borealis*, purplish white in *S. velum*.

Pelseneer[6] has shown that *Solenomya* has a very primitive form of gill and Mitsukuri[7] had previously studied the gill of

Nucula and *Yoldia* and showed also the primitive condition of the gills in these genera. Pelseneer has united *Solenomya*, *Nucula*, *Leda* and *Yoldia* in an order under the name of Protobranchia, forming the lowest order in Lamellibranchs. It is interesting to observe that, while other genera of this low order vary greatly among themselves and from each other, *Solenomya* remains fixed in character despite the wide variation in size among the species and their wide distribution in space.

With the exception of the incomparable work of Deshayes,[2] that of Stempell[11] and Pelseneer[8] on *Solenomya togata* and that of Drew[3] on *Solenomya velum* there have been very few observations on the anatomy or habits of the living creature, and most of the records are derived from accounts of previous observers, and these were in many cases incorrect. Gould says the foot of the animal protrudes behind, whereas it protrudes in front; that the edge of the mantle opening is fringed and that two of the fibrils are larger than the others, which is incorrect, as regards our species and that the palpi are triangular, whereas they are long, narrow and semi-tubular. Stimpson's description, though brief, is without error. Professor George N. Perkins,[9] in his "Molluscan Fauna of New Haven," a memoir teeming with details concerning the soft parts of Mollusca and their habits, has given one of the best accounts of *Solenomya* ever published in this country. Carpenter, in his lectures on Mollusca, says the mantle is closed in front, whereas it is wide open, and that there is a tail on each side of the external opening. Cooke mentions the two long tentacles springing from the siphonal orifice. Stempell figures in *S. togata* two papillæ much longer than the other siphonal processes.

There is a puzzling discrepancy in the figures of the siphonal tentacles of *Solenomya togata* as portrayed by various authors. Philippi[10] figures *S. togata* with short siphonal papillæ resembling a rosette. Pelseneer,[8] in his contributions to the study of Lamellibranchs, figures this species without a trace of long siphonal tentacles. In his introduction to the "Study of Mollusca" he gives a figure of *S. togata* with a pair of siphonal tentacles 17 mm. long. This figure is however copied from Deshayes'[2] "Natural History of Mollusca." It is probably

from this figure that Carpenter, Cooke and others have derived the long tail or tentacle mentioned by them. The excessive length of the two tentacles figured by Deshayes suggests the curious retractile tentacle in *Yoldia limatula* as described by Brooks.[1] A careful examination of *S. velum* however showed no trace of such a sense organ. Stempell,[11] who studied this species at Dohrn's laboratory and published an account in 1899, figures two siphonal tentacles 3 mm. in length. Are these differences the result of variation? Is there more than one species of *Solenomya* in the Mediterranean? Have the drawings been made from alcoholic specimens? The mystery can only be cleared up by a study of the living creature.

In the following observations of *Solenomya velum* I have been greatly indebted to Mr. William F. Clapp, of Cambridge, and Miss Marjorie Newell, of Gloucester, for the material herein described. Mr. Clapp brought me thirty-nine living specimens of *S. velum*, young and nearly full grown. These were collected April 27, on Round Flat, a sandy mud area in Duxbury Bay. They were all buried from six to nine inches below the surface. Mr. Clapp believed they were simply inhabiting abandoned worm holes. Although powerful diggers he thinks they ordinarily dig but an inch or two deep. If they do dig to the depth he found them he believes they must occupy the same burrow for a considerable length of time, for in every case he observed the sides of the hole were discolored, closely resembling worm holes. I placed three specimens in a jar of sandy mud and they soon burrowed to the bottom of the vessel, leaving three sharply defined round holes on the surface of the mud. Drew says: "*Solenomya* lives in rather hard mud, frequently very sandy mud, and, I think, keeps its burrow more or less open." The holes which Mr. Clapp observed and which he cautiously suggested might be abandoned worm holes were undoubtedly holes made by *Solenomya*. Verrill says in regard to *S. velum* that it is "occasionally found burrowing in the pure fine silicious sand near low-water mark, about two inches below the surface, but its proper home is in shallow water beyond low-water mark, and it is perhaps most abundant when there is mud mixed with sand and it also lives in soft mud."

The manner of burrowing is peculiar. In one case an individual buried itself siphon end downward, and for three days remained in this position with its disk-like foot level with the surface of the mud. In every case the individual rested on the bottom of the dish, ventral region uppermost and valves widely open, limited only by the closed portion of the mantle which is drawn tense by the distended valves. When placed on the surface of sand or mud it soon pushes itself backward by means of its foot which, assuming a pointed tongue-like shape, is thrust forward and downward lifting the anterior end and thus depressing the posterior end. Others buried themselves and went to the bottom of the dish, where they remained in a horizontal position. Whether they went head first or tail first was not observed. Others buried themselves in the mud and remained out of sight for hours with no burrows communicating with the surface. Drew states that *Nucula delphinodonta* buries itself in the mud with no surface communication. The strong alternate movements of the sides of the siphonal area may be related to the habit of burying itself posterior end downward. As a further proof that *Solenomya* buries itself siphon end downward may be cited the condition of a young *Solenomya borealis* preserved in alcohol for dissection. I found adhering to the anterior end of the shell close to the margin three colonies of a species of sponge. Here was an evidence that the creature had not only been buried siphon end downward, but that the anterior end had been slightly protruded above the level of the sand and had remained in that position long enough for the accumulation of foreign growth. The periosteum is so exceedingly smooth and polished, and the creature is so active in its swimming habits that the adhesion of foreign growth would hardly be looked for.[1] An examination of a number of shells showed little evidence of wear or burial at either end. The pedal opening is so large and the activities of the foot are so incessant that more water is admitted anteriorly than posteriorly and the siphonal opening has little to do so far as conveying water and food to the gill cavity is concerned.

[1] Dr. Drew informs me that in digging this species from the mud many of them float on the surface of the water, the periosteum repelling the water as if oiled.

If burrowing posterior end downward is a common habit of *Solenomya* it forms an exception to all lamellibranchs that burrow. So far as I know all lamellibranchs that burrow in sand, mud, wood or stone penetrate the substance anterior end first, and in that position they rest. Indeed the foot is the primary instrument used in effecting this penetration. *Solenomya* is unique in this respect.

As before stated the creature, resting on its back, thrusts out its foot in a pointed shape, presses the bottom of the dish, then immediately withdrawing it at the same time expands the fimbriated disk which unfolds in a graceful manner, and in that expanded condition swings back and forth a few times in the pedal opening which is widely distended. The overlapping periosteum then folds abruptly within the shell, which closes at the same time as the thick foot is drawn in between the polished walls of the periosteum. The creature then falls over on its side and remains in that position until the valves again open which is almost immediately. The method of swimming has been accurately described by Drew. The act consists in thrusting out the foot, promptly expanding it and then suddenly withdrawing it, at the same time closing the shell and expelling the water from the siphonal end. These motions are often repeated a number of times without the animal moving at all. When these movements are made with sufficient vigor, however, the animal seems to leap or dart in the water, anterior end forward, going three or four times the length of the shell at each leap.

Stimpson says: "The thinness of the shell enables the animal to make surprising leaps and I have seen it leaping or swimming about the water for some time without touching the bottom. The leap is performed by suddenly drawing in the umbrella-shaped foot, at the same time that water is expelled from the posterior opening by the closing of the valves." I counted the number of darts made by different individuals and, though the specimens I had were probably enfeebled by their long transportation in a small glass vessel, I found the following result—22, 24, 31 and 36 darts respectively were made before the creature fell to the bottom of the dish. The 36 darts were made by a young individual. The darts were vigorous, and were made at the rate of 90 to 100 a minute.

The violent activity of the animal in its rapid darting through the water, the repeated thrusting out of the heavy pedunculate foot and vigorous closing of the valves accounts for the necessity of the continued absorption of food as indicated by the rapid accumulation of flocculent fœcal matter in the dish.

The thirty-nine specimens, young and nearly adult, collected April 27, were placed in a white enamelled pan and showed no signs of increased enfeeblement for a week or more. The water was changed often. At the end of three weeks they were all dead, the young ones surviving the longest.

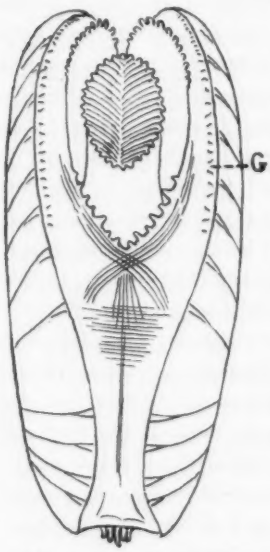


FIG. 1. View of animal from below.

The individuals showed no sensitiveness to the obstruction of light, nor did they align themselves in any special way in relation to the lighted window. They did not seem to have the sensitiveness of other lamellibranchs; the jarring of the table did not cause them to close, though agitating the water or touching them with a needle ever so slightly prompted them to close their shells, which remained closed for a few seconds, when they slowly opened and repeatedly thrust out the foot. In fact they became very lively after agitating the water.

As one views a specimen from below with the valves wide open and the closed part of the mantle stretched like a drumhead from the margin of the periosteum, the calcified portion of the shell is hardly in view. The periosteum, extended as it is when the valves are open, shows no marked shoulder or channel at its junction with the thin membranous mantle, though in section a slight break is seen. The pedal opening is wide and extends backward nearly to the center of the body, where the free mantle edges come together, forming an elongated oval opening when distended, and through this opening

the thick stocky foot is seen occupying the whole space (Fig. 1). At the point of junction of the two sides of the pedal opening there are crowded together muscle fibers, producing a silvery appearance, and at this point muscle fibers that run parallel to the borders of the pedal opening—which Drew described as in the nature of sphincter muscles—cross to opposite sides and continue their course posteriorly. May not the origin of the peculiar sharply defined cruciform muscles in *Solecurtus*, *Tagelus*, *Macoma* and *Tellina* be traced to these separate crossing muscle fibers in some primitive lamellibranch like *Solenomya?*

FIG. 2.

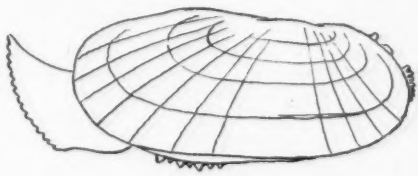


FIG. 3.

FIG. 4.

FIG. 2. Pedal opening showing white area, *W*.
FIG. 3. Side view of animal.
FIG. 4. Papillæ on margin of pedal opening.

The edge of the pedal opening is at times reflected and at its junction in the median line a few blunt papillæ or tubercles appear along the edges to nearly half the length of the opening. These are so aligned that when the two edges come together in closing the tubercles interlock. In Fig. 2 the tubercles are seen with the pedal opening nearly closed. They vary in size, are contractile and stand out at a sharp angle from the border so that they are distinctly seen when the animal is viewed from the side (Fig. 3). Until nearly adult these tubercles are translucent, but in the oldest specimens they become opaque white, resembling white kid, and appear hard and horny until touched when they partly retract. They are covered with a transparent epithelium (Fig. 4). This peculiar whitening not only covers the tubercles but whitens the mantle border from which they spring. A blotch of white is also seen between the tubercles and the keen edge of the periosteum, which at this point is also

touched with white. Even the base of the foot behind has a small blotch of white showing only when the foot is greatly elongated. This coloration in all exposed parts of this region is certainly a curious feature. It is as sharply defined and localized as if one with a paint brush had delicately touched with white every exposed part of the animal within this limited area. It is true the external shell is also touched with white at the anterior end. In the young a dot of white is seen in the first three or four transparent interspaces of the free periosteum. In older specimens these white spots are drawn out in broken lines; not in all specimens, however. In older specimens anteriorly the

FIG. 5.

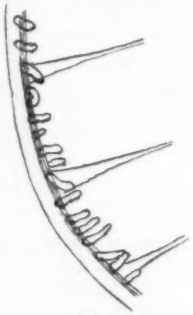


FIG. 6.

FIG. 5. Glands from side of pedal opening.
FIG. 6. Glands from anterior mantle margin.

whitening is sometimes seen on the dorsal side of each rib. This peculiar coloration is apparently correlated with the attitude of the animal in nature. Resting on its back the ventral region of the animal shows this whitening. Are the white markings at the anterior end of the shell another indication that the creature more often or habitually buries itself siphonal end downward?

Along the anterior half of the mantle, as it springs from the edge of the periosteum, are seen a number of what appear to be short white lines with glandular enlargements. They begin a little in advance of where the mantle parts, forming the pedal orifice, and are separated by a considerable space (Fig. 5), becoming crowded toward the rounded anterior border (Fig. 6). They spring from just under the edge of the periosteum and

point in the general direction of the median line, but are disposed irregularly in the membrane, some inclined forward and others backward, but most of them are at right angles to the mantle border (Fig. 1, G). Under a higher power these bodies have the appearance of setigerous follicles. Stempell figures one in his memoir of *S. togata* as a simple alveolar border gland. His figure shows a much more bulbous gland and the thread-like extension is not shown as in those here figured (Fig. 5). It was some time before I determined that there was no protuberance beyond the surface of the mantle, so strong was their resemblance to setigerous follicles.

FIG. 7.

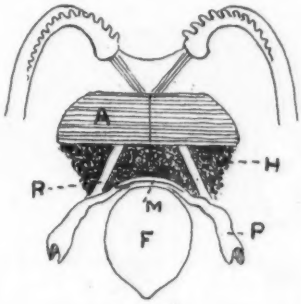


FIG. 8.

FIG. 7. Anterior view of animal.

FIG. 8. Anterior end from below: *A*, anterior adductor muscle; *F*, foot in section; *H*, hepatic follicles; *M*, mouth; *P*, palp-appendages; *R*, pedal retractors.

The margin of the mantle becomes thickened as it borders the anterior end of the shell and ends abruptly just before the shells meet dorsally. Just in front of where the shells meet above are two short unpaired tentacles or tubercles in the median line and then ten or more on the edge of the rounded anterior edge of the mantle on each side diminishing in size, however, toward the ventral region, where they cease (Fig. 7). Writers have described the whole free edge of the mantle as bearing these papillæ and even shown them in figures. Looking at this region from below the abrupt termination of the thickened mantle margin and the papillæ are shown (Fig. 8). A triangular muscular layer seems to connect the two thickened portions of the mantle dorsally. Also a narrow band of fibers runs from the

abrupt terminations of the mantle converging centrally. Fig. 8 shows the relation of these various parts in front of the anterior adductor.

As the animal becomes enfeebled the mantle (or, what would be a more proper name, the ventral membrane) ruptures, exposing the dark-colored gills below. There is a median suture in this ventral membrane and in one rupture the suture became dislocated, showing there was a strain upon it. In another case a small rupture appeared on each side of the median suture. That this membrane limits the expansion of the valves is shown by cutting the membrane, when the valves immediately open

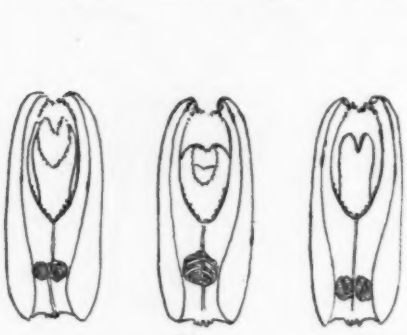


FIG. 9. FIG. 10.

FIG. 9. Various attitudes of foot, and ruptures in ventral membrane.
FIG. 10. Dorsal view of hinge margin.

wider in much the same way as when the adductors are severed in other lamellibranchs. Fig. 9 represents the various ruptures, and at the same time illustrates different attitudes of the foot. In the adult the pedal opening extends back nearly half way to the center of the body. In the young it extends a little over a third back. Viewing the shell from above, the exposed ligament presents some curious features which I have tried to represent in Fig. 10. In the vicinity of the umbones a narrow elongated wedge-shaped substance, black in color, is seen, behind which a brown oval ligament appears, split at its posterior end in which is wedged a white substance. From the junction of the two valves anteriorly a narrow wedge-shaped substance narrowing posteriorly is peculiar in being a polished white. In the young

the periosteum is light yellowish, growing darker as age advances. The hypobranchial gland appears as a mass of globular cells containing short rod-like bodies. A number of graceful attitudes are shown in the movements of the pedal disk which I found difficult to figure. The fimbriated edge shows as distinct short tentacles w th grooves running down outside, corresponding to the tentacles and corresponding lines or grooves in the disk running to the median line resembling a diminutive actinoid coral *Fungia*. At other times the edge of the disk appears serrated. These number thirty in all (Fig. 11). In the young these resemble long papillæ.

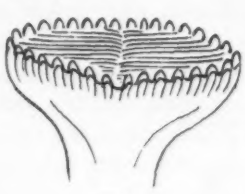


FIG. 11.

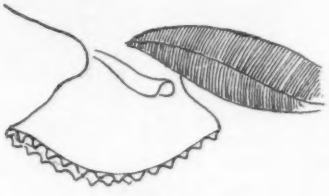


FIG. 12.

FIG. 11. Dorsal view of pedal disk.

FIG. 12. Side view of foot, showing palp-appendage and branchia.

The gills are most peculiar in *Solenomya*. As Pelseneer has shown, there is only one gill on each side having an upward and downward series from a central base. This line dividing the upper and lower series runs from the dorsal anterior portion of the gills to nearly a ventral point posteriorly (Fig. 12). The filaments are distinctly separate and are beautiful in their regularity. The filaments are arranged on the support from which they spring directly opposite each other. Each filament is supported by a chitinous rod in one series, slightly bent at the end—firm yet elastic. The same chitinous framework is seen in the gills of *Nucula proxima* and *Yoldia limatula*, as shown by Mitsukuri, Drew and others. Mitsukuri,[7] in describing the chitinous framework supporting the gills in *Nucula proxima*, says: "Whether it is really formed of chitin I do not know, but as previous writers have described the substance as of that nature it will be convenient to use the term 'chitinous support' for the present." The gills are colored a light brownish purple, their posterior terminations are free and lighter colored (Fig. 13).

A view of the posterior end of the animal reveals a single long siphonal opening which often changes its outline, at times being an oblong, oval slit, but usually the upper part, corresponding to the anal opening, is much larger, giving the aperture a double-gourd-shaped form, the upper bulb being larger. Above the anal region, just where the two shells meet, is an unpaired tubercle, or tentacle which is contractile. Stempell figures a similar

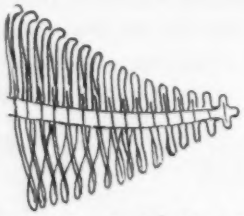


FIG. 13. Termination of branchia.

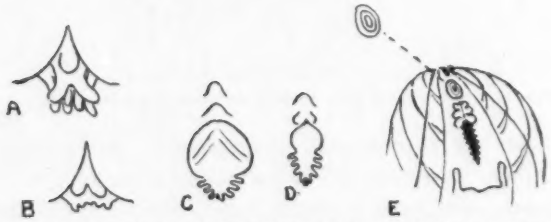


FIG. 14. Siphonal opening: *A*, *B*, from above; *C*, *D*, from behind; *E*, from behind at an angle.

tubercle in *S. togata*, and in another form to be described later a very long tentacle is seen. Just below this tentacle a pair of blunt tubercles arise which are very contractile and appear at times as two and even three pairs of tubercles. The sides of the anal opening are without tubercles. The narrow branchial opening is bordered on each side by from six to eight short tubercles or papillæ hardly varying in size. These are also contractile. A few attitudes of the siphonal opening are shown in Fig. 14.

A view of the siphonal opening from the side shows no protrusion as in the siphonal expansion of siphonated lamellibranchs. Indeed there is no appearance of a siphon, simply an

opening with papillæ about it and these quite unlike the long pointed papillæ of the siphonated lamellibranchs or even the truncate papillæ of *Saxicava*, though morphologically they are the same. On viewing this region from above the papillæ appear bunched together or spread apart, the result of the sudden dilatation or partial contraction of the opening.

The sides of the siphonal opening are in constant motion in and out, though this motion is alternate and rhythmic. The proboscidiform foot swings from one side to the other within the shell, like an elephant's trunk and this motion may be related to the alternate siphonal movements; this correlation, however, was not observed.

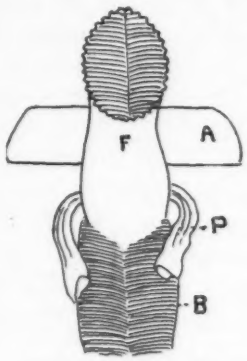


FIG. 15. Anterior end from below: *A*, anterior adductor muscle; *B*, branchia; *F*, foot; *P*, palp-appendages resting on branchia.

Just below the siphonal opening a slight depression is seen on the mantle on each side connected by a transverse depression forming a symmetrical figure. Stempell shows a marking of the same nature in *S. togata* in the form of an angle with the apex downward.

A view of the animal as it rests in the usual position on its back with valves widely apart will reveal the attitude and behavior of the palpi (Fig. 15). Cutting the ventral membrane will give a clearer view of these parts. The palpi are adherent for a short distance to the side of the foot but are free beyond, and extend backward and downward. These organs are long, slender, semi-tubular with ends much larger and the whole structure delicate and diaphanous. No line or angularity in section indicates in any way that this semi-tubular appendage has come about by the adhesion of two palpi. The palpi of other lamellibranchs are more or less triangular in shape with their upper edges partly united. In *Solenomya velum*, the long tube-like appendages encircle the base of the foot with their dilated extremities resting directly on the compact sides of the

gill plates, and in this position are in gentle and constant motion with occasional side jerks. After watching these browsing movements for awhile one becomes convinced that the behavior of the ends of the palpi is that of feeding. Even the peculiar jerks at times suggest that some larger morsel has been caught. The protean shapes the appendage assumes are shown in Fig. 16. The strong ciliary action of the gills is continually sweeping along particles of organic matter toward the feeding appendages which gather the stuff and convey it by ciliary action to the mouth. The movements of these appendages may be distinctly seen through the ventral membrane and at no time are they at rest. The continual ingestion of food is indicated by the great quantity of excreta which is voided. In this connection it is interesting to note that Drew[6] in his memoir on the life habits of *Yoldia limatula*, another member of the Protobranchia, describes long appendages to the palpi which extend backward beyond the posterior end of the shell and rest on the sand. These appendages are semi-tubular and being ciliated "rapidly elevate the mud which is full of living organisms and finally pass it between the palpi," and thence, of course, to the mouth.

FIG. 16. Ends of palp appendages.

Mitsukuri, having described the palpi in *Nucula proxima*, says: "At their posterior end there are two remarkable structures. One of these is a hood-like structure which is the posterior prolongation of the united upper edges of the inner and outer palpi. The other, lying immediately below the first, is a long tentacular appendage. It is a hollow tube, open, however, along a line on its posterior aspect, and having its cavity continuous with the space between the two palpi. As it has been seen protruded with the foot outside of the shell, and since, in alcoholic specimens, a great deal of dirt and sand is found along its length and between the palpi from its base to the mouth, it is no doubt a food-procuring organ, probably sending a constant stream of nutritive matter to the mouth by means of its cilia. It is interesting to notice in connection with this appendage that in *Nucula*, the gills, unlike those of ordinary lamellibranchs, must be practically useless for obtaining food, as will be evident

from the following description of them," etc. Drew[4] finds these palp-appendages in *Nucula delphinodonta* functioning as food collectors. The creature is immersed in mud out of sight and only when placed in shallow mud was he able to observe its behavior. The gills are certainly very small in *Nucula*. In *Solenomya*, however, the gills are very bulky, filling nearly half the mantle cavity, are highly ciliated and as we have seen the palp-appendages rest directly on their anterior surfaces. There can be no question that these appendages in *Solenomya* are strictly homologous with the palp-appendages in *Yoldia*, *Nucula* and allies. The gills, however, are the food accumulators from which as we have seen the palp-appendages, collect the material for nutrition. The mouth is difficult to make out in the living creature. In two instances I have observed a slightly brownish line marking the position of the mouth.

While there are many features in common between *Solenomya* and the other members of the order Nuculidæ as seen in the posterior position of the umbones, the primitive gills, the palp-appendages, the fimbriated disk-like foot, the highly polished periosteum; the absence of true palpi in *Solenomya* might be considered an evidence that this peculiar form stands lowest in the order Protobranchia.

Some years ago Miss Newell discovered at Annisquam a colony of what was supposed to be *Solenomya velum*. A few were brought home alive, but circumstances were not favorable at the time for detailed study and only a few drawings were made. The shell is apparently identical with that of *S. velum* herein described, yet the character of the siphonal opening or rather the appendages surrounding that aperture are widely different. The single siphonal orifice is ano-branchial as in the other species of *Solenomya*. Above this orifice is a very long unpaired tentacle with broad base. On each side of the anal region is a broad flap bearing five tentacles, the lowest one small, the next one long slightly curved upward with three minor twigs springing from its side, the next three smaller and diminishing in size. Below this process is a wider flap supporting nine tentacles of nearly uniform length. Between the anal and branchial processes viewed from the side is a white band! I hesitate giving these

details for fear some systematist will instantly announce a new genus or family. Nevertheless, no figure nor description of *Solenomya* yet published has shown anything approaching these remarkable processes. The unpaired dorsal tentacle is short in *S. togata* (Fig. 18), even shorter in *S. velum* (Fig. 19), while in this form (Fig. 17) it is exceedingly large with a broad base. The

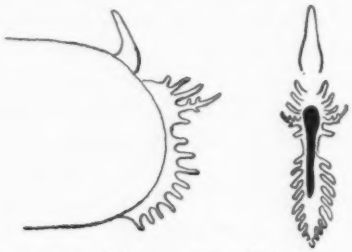


FIG. 17. *Solenomya* sp.? Annisquam.

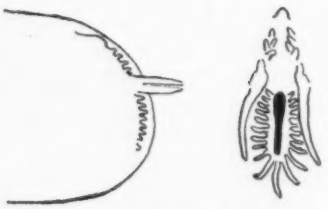


FIG. 18. *Solenomya togata.*

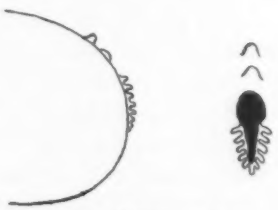


FIG. 19. *Solenomya velum.*

pedal opening shows the same tentacles starting from the posterior end of the opening. The same alternate movements of the siphonal opening were observed. The shell as before remarked differed in no respect from that of *S. velum*, and I am puzzled at the remarkable difference in structure of the siphonal opening. The question arises whether the Annisquam form may not be the young of *S. borealis*. This can only be decided by securing a living specimen of this rare form.

Professor Drew,[5] in his observations on the habits, anatomy and embryology of members of the Protobranchia, alludes to the great diversity in structure and concludes that the protobranchia have been derived from a primitive type. The resemblance he finds in certain stages of the embryo of *Yoldia* and

Nucula to those of certain other mollusks is interesting in the fact that these mollusks are in every case low and aberrant forms. He says: "The most striking peculiarities in the development are connected with the formation and disappearance of the tests. Outside of the group, so far as I have been able to learn, *Dondersia* is the only other mollusk whose embryos are known to be provided with similar tests."

Dondersia is now regarded as belonging to the Amphineura, but for awhile was looked upon as related to the worms. Drew remarks that the young embryos of *Dentalium* bear certain resemblances to the embryos of *Dondersia*, *Yoldia* and *Nucula*. Here again a resemblance is seen to an aberrant group of mollusks whose affinities were for a long time obscure. Drew also says that a somewhat similar resemblance is noticeable in the case of the embryo of *Patella*. Again a resemblance is found to a group whose characters are archaic. Dall, in speaking of the Docoglossa, says the various forms manifest what may be termed a peculiar persistency of immaturity when compared with other groups of gasteropods. Korschelt, Patten, Fisher, Lankaster, Pelseneer and others testify to the primitive characters of *Patella*. In my paper on "An Early Stage of *Acmæa*," I have collected a number of extracts from the above authors in regard to these low characters.

Pelseneer agrees with Stempell that the characteristic features of *Solenomya* represent the oldest living group of the lamellibranchs. He calls attention to the absence of a protractor pedis, extensive overgrowth of ventral mantle edges, peculiar development of the branchi-anal siphon, the form of shell and absence of interlocking teeth, with position of ligament, remarkable overlapping of periosteum, prismatic structure of limy shell, rudimentary form of mouth lips and mouth appendages, almost without winding of intestines, excessive elongation of the ventricle, the presence of pericardial glands, position, size and structure of gills, separation of kidneys without cross communication.

Interesting features will doubtless be revealed when the embryology of *Solenomya* shall have been made known, and with the abundance of one species on our coast, *S. velum*, we hope before long light may be thrown on the subject.

Appendix—Notes on Living *Solenomya borealis*.

Since the above observations were made on *Solenomya velum*, and the matter in type, the opportunity has been given me of studying living specimens of the large species *Solenomya borealis*. My friend, Major John M. Gould, collected a number of living specimens of various sizes from a young one, measuring 9 mm., to full-grown individuals, measuring 83 mm. These were obtained from dredgings in six to seven fathoms of water by a huge dredging machine engaged in deepening a channel in Portland harbor, Maine. The work of collecting, as may be imagined, was done under desperate conditions. Jumping in between avalanches of mud and water, glancing, raking and jumping out again! In this rapid reconnaisance Mr. Gould observed that the creatures were buried in holes and got the impression that they were buried posterior end downward, the broad, light-colored disk-shaped foot projecting from the holes. This attitude, as before remarked with regard to *S. velum*, is contrary to the behavior of all lamellibranches that bury themselves wholly or partially. I am still in doubt about the matter. It is interesting to note, however, that when the creature was placed on coarse material it behaved in precisely the same way as *S. velum*. In every case resting on its back it thrust out its foot, raised the anterior end of the body as if endeavoring to thrust the posterior end into the mud. In this connection, it is interesting to observe that the anterior ends of the shells of older specimens were covered with films of slime while the posterior ends were clean and polished.

In only one instance has the animal been seen to swim or dart through the water though both young and old were specially observed for this behavior. The foot was often thrust out as in *S. velum*, but not so often nor with such energy. No lateral swing of the foot was observed. The periosteum appeared oily as in *S. velum* and repelled the water, and Dr. Drew informed me that this feature was so marked in *S. velum* that when he dug them from the mud they sometimes floated on the water.

The character of the siphonal end upon which marked specific differences will be established was quite different from *S. velum* and it can now be positively stated that *S. velum* and *S. borealis*

are distinct species. It is surprising, however, to see how closely they resemble one another (Fig. 20). At first sight *S. borealis* seemed to be an enlarged *S. velum*. The color of the foot and ventral membrane varies. In *S. borealis* the foot is a light brown with a tinge of purple, darker on the ventral keel, with a darker blotch near the ventral portion. The papillæ on the edge of the

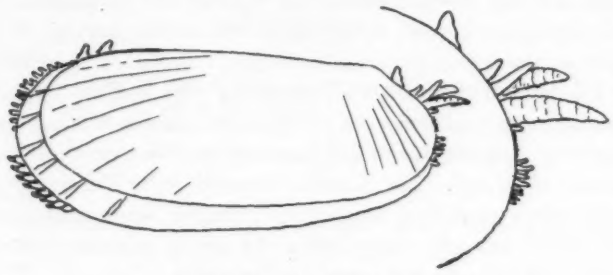


FIG. 20. Side view of *S. borealis*, natural size.

pedal disk were longer and sharper than in *S. velum* though the same in number. The tubercles on the edge of the mantle opening were the same in number and position, standing out at right angles from the ventral membrane. Their color, like the membrane, was brownish, the tips of the tubercles being dark brown. No trace of a white pigment-like color on ventral membrane

FIG. 21.

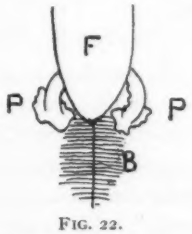


FIG. 22.

FIG. 21. Siphonal opening.

FIG. 22. Anterior end from below: *F*, foot; *P*, palp-appendages resting on the branchia; *B*, branchia.

or tubercles was observed in either young or old. The palpi were not so long though the ends were more foliated (Fig. 22). Their movements were not so active. The palpi rested on the anterior portion of the gills and were evidently engaged in secur-

ing food as described in *S. velum*. Alternate movements of the side of the siphonal opening were observed as in *S. velum*. The anterior aspect of the creature was much the same, the two median dorsal tubercles, one behind the other, and the anterior edge of the mantle bearing papillæ. The posterior aspect was quite different from *S. velum* and resembled more closely *S. togata* of the Mediterranean as figured by Stempell. The resemblances are seen in the large size of the median dorsal tubercle, the few small tentacles in pairs, succeeded by a pair of very long tentacles, then a number of very minute tentacles bordering the upper edges of the siphonal opening followed by a number of tentacles increasing in size to the lower portion of the opening (Fig. 21). All of these tentacles were retractile, the upper series appearing like round tubercles when contracted. The longer tentacles surrounding the orifice were in constant action, a bending-in movement as if grasping.

In the collection of fifteen specimens of *S. borealis* the individuals varied in length from 9 mm. to 83 mm. Omitting the smallest specimen the others were easily arranged in four distinct series of sizes as follows:

1st Series.	2d Series.	3d Series.	Full Grown.
24 mm.	39	57	73
29 mm.	40	59	77
31 mm.	44		77
31 mm.			78
			83

Twenty-eight specimens of *S. velum* from Sandwich were in the same way readily grouped in series. One measured 7 mm. in length, 6 averaged 10 mm., 8 averaged 12 mm., 5 averaged 13 mm., 6 averaged 15 mm. and 3 averaged 19 mm. From this one might infer that *Solenomya* is not an annual.

In conclusion I wish to say that this brief examination of the external features of *S. borealis* has convinced me that my supposition that the Annisquam individual might be the young of *S. borealis* is incorrect. Furthermore that a study of a more advanced specimen of *S. velum* at Woods Hole showed a nearer approach to the Annisquam specimen. I have never seen a minute specimen of *S. velum* and Professor Drew informs me that he has never found one with eggs. The protoconch of *Solenomya*

will show interesting features and the embryology of this low form will be of great importance.

EXPLANATION OF FIGURES.

Note.—Only the largest specimens averaging 19 mm. were studied. The various degrees of magnification of the figures here given may be understood in a general way by realizing that the entire length of the animal was 19 mm.

BIBLIOGRAPHY.

1. **Brooks**

'74 On an Organ of Special Sense in the Lamellibranchiate Genus *Yoldia*. Proc. Am. Asso. Adv. of Sci., 1874.

2. **Deshayes**

'44–'48 Histoire naturelle des Mollusques. (Exploration scientifique de l'Algérie.) Paris, 1844–1848.

3. **Drew**

'00 Locomotion in *Solenomya* and its Relatives. Anat. Anzeiger, XVII. band, No. 15, 1900.

4. **Drew**

'01 Life History of *Nucula delphinodonta*. Quart. Journ. Micr. Sci., XLIV., 1901.

5. **Drew**

'99 Some Observations on the Habits, Anatomy and Embryology of Members of the Protobranchia. Anat. Anzeiger, XV., 1899.

6. **Drew**

'99 *Yoldia limatula*. Mem. Biol. Labor. Johns Hopkins, Univ., IV., 1899.

7. **Mitsukuri**

'81 On the Structure and Significance of Some Aberrant Forms of Lamellibrancheate Cills. Quart. Journ. Micr. Sci., XXI., 1881.

8. **Pelseneer**

'91 Contribution à l'étude des Lamellibranches. Arch. de Biol., XI., 1891.

9. **Perkins**

'69 The Molluscan Fauna of New Haven. Proc. Soc. Nat. Hist., Vol. 13, 1869.

10. **Philippi**

Über das thier von *Solenomya Mediterranea*. Arch. Naturg., Jg. 1, Vol. I.

11. **Stempell**

'00 Zur Anatomie von *Solenomya togata*. Zool. Jahrb. (Anat. u. Ontog.), XIII., 1900.

A CHEMICAL SIGN OF LIFE.[1]

(From the Laboratory of Biochemistry and Pharmacology, The University of Chicago; and Marine Biological Laboratory, Woods Hole, Mass.)

SHIRO TASHIRO.

From the earliest time to the present, our criterion of life has been connected with the changes which are brought about by death. The locomotive power of living matter is one of the first things that disappears when it dies. Thus the idea of movement erroneously led the simple minds of our ancestors to believe that wind, fire, thunder and water had life. By close study of the locomotion of living matter, however, we have gradually traced this part of living phenomena to irritability of tissue—the property characteristic to living tissues only. This protoplasmic irritability is not only the potential of living matter, to give rise to physical changes when a stimulus is applied to it, but the fundamental and the only characteristic which is common to all the living as long as they possess the power to perform their own functions. And it is this property, according to Professor Mathews,[2] that not only is the most probable point of attack of natural selection but is also one of the main factors which determines phylogenetic development of organisms.

The presence of this irritable property in tissue—the universal sign of life—cannot easily be determined in all living tissues. The most common way of determining irritability is the physical changes brought about by a stimulus. This physical change, however, is not an unfailing indication of a response of the tissue against stimulation. Several tissues in the animal, such as nervous tissues, and an abundance of examples in the plant kingdom, do not manifest at all any visible change, when stimulated. Not only those living do not show such mechanical

[1] I wish to thank Dr. F. R. Lillie, through whose kindness I was occupying a table in the Marine Biological Laboratory, Woods Hole, Mass., at the time when the apparatus for these experiments was made.

[2] Mathews, *Amer. Naturalist*, XLVII., p. 94, 1913.

changes while functioning, but several non-living matters constantly undergo physical changes when disturbed by external conditions. Therefore it has been customary for the biologist to decide whether or not matter is living by several other accompanying changes during the performance of a function. Such changes have been measured by rise of temperature, production of CO_2, histological variation before and after the stimulation, and electrical response. None of these functional changes when taken individually can be considered as a specific sign of life.

Although Herzen claims that under certain conditions of local narcosis, the nerve fiber may give an action current although no muscular contraction follows, and O. B. Ellison recently demonstrated by the use of cinchonamine hydrochloride the absence of negative variation without abolishing excitability, yet according to Waller the presence of life can be demonstrated by an electrical change. In his admirable book on the "Signs of Life,"[1] he states that chemical change is a sign of life and an electrical change is a sign of a chemical change; therefore the electrical change is the sign of life. It is very interesting to note in the case of a dry seed, he could measure equally well quantitatively the different electrical changes according to the different ages which characterize the different degrees of vitality of the seed, in spite of the fact that he could not detect any chemical change which usually produces CO_2. He concludes, however, that it is possible, or rather certain that our method of chemical investigation is not refined enough to reveal to us the smallest and most infinitesimal change that may be going on in apparently dry or dormant seed. It was this conclusion of his that suggested to me the desire to make an inquiry to ascertain whether or not I could find some new method by which an easy sign of life may be observed.

When I had constructed a new apparatus,[2] which can detect CO_2 as small as 0.0000001 gram, the measurement of irritability became much simpler, for with it we established a few new facts which deal with the fundamental nature of protoplasmic irritability. The idea that irritability in general is closely associated

[1] Waller, "Signs of Life," New York.

[2] *Am. J. of Physiol.*, XXXII, p. 137, 1913.

with chemical phenomena has been set forth long ago. Professor Mathews, in his paper on "Animal Oxidation," expressed the idea that cellular respiration must be the fundamental process of all organic activity around which all functional phenomena are intimately connected. Later in his study on the action of ether and other anæsthesias on an anaerobic tissue he confirmed his idea that all the substances that affect irritability must necessarily attack the tissue respiration first. With the aid of the new apparatus, the direct evidence for his hypothesis has been brought forth in connection with the study of metabolism of the nerve fiber, in which three fundamentally interesting facts have been established. In the first place, the most excitable tissue of all the protoplasm, the nerve fiber, is constantly undergoing chemical changes, giving off CO_2. In the second place, when this tissue is stimulated CO_2 production is greatly accelerated, giving more than double the amount. Finally, the rate of CO_2 is greatly influenced by conditions such as anæsthesia, which are known to affect tissue irritability, showing a direct relation between respiration and excitability.[1]

Before I had concluded, from these facts, that all the irritable tissues must respire and should give off more CO_2 when stimulated, a crucial experiment was done on a dry seed. Waller's conclusion has been fully realized. A living dry seed not only gives off CO_2 quantitatively proportional to its weight, but also gives off more CO_2 when stimulated, a phenomenon true to living seeds only. These facts enable me to propose a new sign of life, namely, a chemical sign. The criterion is simple. If we are given a tissue which gives more CO_2 when stimulated, the tissue must be alive; it is excitable. A discovery of a remarkably simple method of stimulation made this chemical sign of life much more easily practicable to all living tissues. Simple mechanical crushing of the living tissue is the new method. I have already argued elsewhere[2] that the phenomenon that the nerve gives off more CO_2 when crushed, is due mainly to an extreme stimulation and that it is characteristic of living, excitable tissues only. Therefore without any attempt to settle the question as to how CO_2 production is increased, we can use

[1] *Am. J. of Physiol.*, XXXII., pp. 107–136, 1913.
[2] *Am. J. of Physiol.*, XXXII., p. 121, 1913.

this method as a means to denote protoplasmic irritability, as long as we can abolish this sign of life by rendering the tissue unexcitable. The nerve, dry seeds, including wheat, oats, rice and several other living tissues from animal and plant kingdoms, if treated with ether, or killed, can never be made to produce more CO_2 by crushing, whereas, a tissue however little CO_2 it may produce normally will give off more CO_2 when crushed, provided it is living. From these general findings, I conclude that this method can be used to detect vitality of protoplasm under normal conditions.

The details of this simple means of finding the sign of life are as follows:

The biometer is used. This is an apparatus which has two respiratory chambers, each furnished with a cup in which a drop of barium hydrate can be introduced. With it, the comparative outputs of CO_2 as small as 0.0000001 g. from the two tissues can be estimated simultaneously.[1] Two dry living kernels of the same seed, with equal weights, are chosen. One is placed in the right chamber, and the other is crushed and placed in the left. After the necessary cleaning of the apparatus with CO_2-free air, a drop of the barium hydrate is introduced upon each cup in both chambers. By watching the drop, it would become obvious that the crushed seed is giving off more CO_2 than the uninjured, as indicated by the speed of formation and quantity of precipitate of the barium carbonate. Not only such distinction between two can be observed, within a few seconds, in the case of the tissue, which normally gives off a comparatively large quantity of CO_2, but also dead tissue whether or not it gives off CO_2 without crushing, will never produce more CO_2 when mechanically smashed. In other words the phenomenon of production of more CO_2 by crushing is characteristic only of a living tissue. By killing the seeds, and repeating the experiment, such a conclusion can easily be confirmed.

With these facts, I am proposing a new sign of life, namely, a chemical sign of irritability. It is a measurement of CO_2 due to stimulation. It is not exhalation of CO_2 which is character-

[1] The detail of this apparatus is described in *Am. J. of Physiol.*, XXXII., p. 141, 1913.

istic of life, for there are many dead matters which give off CO_2 continuously, but it is the increase of CO_2 production when crushed which is certainly a phenomenon associated with excitable tissue only. Hence, when a tissue gives off more CO_2 when mechanically crushed, or injured, it is a sure sign that this tissue is living.